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The Contribution of New Neurons to Behavioral Plasticity in the Juvenile Zebra Finch

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ABSTRACT:

The mechanisms by which bilingual humans maintain and acquire memory for multiple languages are unclear. Although monolinguals exhibit left dominance for speech perception and production, the lateralization underlying speech perception and production in speakers of multiple languages is debated. Songbirds, such as zebra finches (*Taeniopygia guttata*) are often used in the laboratory to study language and speech acquisition in humans. In order to mimic the sequential learning experience of bilinguals, we can manipulate the auditory environment of zebra finches by exposing them to two tutors at different points in development. Previous work in the lab found mirrored lateralization of first and second tutor memories in the caudomedial nidopallium (NCM), the avian analog of the mammalian auditory cortex (Olson et al., *in review*). The more song elements birds retained from their earlier tutor, the more right-dominant neuronal activation is observed in NCM in response to the first tutor song. The more birds learned from the later tutor, the more left-dominant neuronal activation in the NCM in response to the second tutor song. This suggests that the right hemisphere may be resistant to changes in the auditory environment, resulting in a more permanent representation of early-learned song, whereas the left hemisphere may be more successful at adapting in response to a novel auditory environment and thus encode the later tutor song. Because new neuron addition is left-lateralized in the NCM, this raises the possibility that greater new neuron addition to the left NCM facilitates successful adaptation to a novel auditory environment. The HVC (proper name), a region involved in the motor pathway for song production, also recruits new neurons; greater new neuron addition in the HVC in adulthood is related to increased song stability. In this thesis, we examined the

relationship between new neuron addition in NCM and HVC and maintenance of the first tutor song over the course of development and successful acquisition of a second tutor song. We found no relationships among song maintenance and learning and new neuron density in HVC. However, in NCM, new neuron addition in left and right lateral NCM was correlated with more successful learning of second tutor song, and new neuron recruitment in the neural representation for second tutor song in the left lateral NCM was correlated with greater similarity between tutee and second tutor at adulthood. Our results demonstrate that neurogenesis in NCM may underlie successful acquisition of a second tutor song in development.

1. INTRODUCTION

The ability to speak facilitates our existence as humans. Through speech, we cultivate and sustain relationships with other individuals, ask questions and gain knowledge about our environment, and communicate our thoughts, ideas, and opinions to others, leaving a mark of our identity on the world that can remain once we are gone. Life without language is isolating and can sequester an individual from the ones they love, as the ability to communicate is essential not only for identity development, but also in understanding where one aligns with others in their environment. Individuals with speech disorders, including aphasia, stuttering, and autism, demonstrate deficits in their ability to communicate with others, which can result in detrimental social and behavioral outcomes. In order to better understand how speech is altered through these disorders, it is necessary that we understand how human speech is developed and maintained.

The Zebra Finch (*Taeniopygia guttata)* as an Animal Model for Human Speech Acquisition

Zebra finches (*Taeniopygia guttata*) can be used to study language and speech acquisition in humans, as songbirds and humans share behavioral, genetic, and anatomical similarities, and the trajectory of auditory and vocal development is highly similar. The trajectory of song learning in the zebra finch parallels that of human infants; both are composed of three general phases (Doupe and Kuhl, 1999; Bolhuis and Moorman, 2015, Figure 1). The first phase is a sensory learning phase in which a template, or neural representation for language or song, respectively, is learned and encoded. Human infants acquire the sensory templates for speech sounds from adults in their environment, usually their parents, from birth to about four months of age (Doupe

and Kuhl, 1999). Zebra finches learn song from a single conspecific tutor, usually their father. The neural representation for this tutor song memory is encoded between 20 and 35 days post hatch (dph), in a region known as the caudomedial nidopallium (NCM) (Doupe and Kuhl, 1999). The second phase is a sensorimotor phase in which vocalization develops through practice. At about seven months of age, human infants enter a babbling stage, in which they begin to vocalize, but there is no real pattern or meaning underlying these sounds. Similarly, between 35 and 90 dph, juvenile zebra finches are also practicing vocalizing, potentially using the encoded tutor song template as a model (Doupe and Kuhl, 1999). Distinct song syllables, or individual units of song, and syllabic patterns represented in the zebra finch's adult song are beginning to emerge, and the song begins to sound more like the tutor's song (Doupe and Kuhl, 1999). For both humans and zebra finches, the sensorimotor phase is hypothesized to involve auditory feedback of practiced vocalizations and matching these vocalizations to the stored speech or song templates (Doupe and Kuhl, 1999; Achiro and Bottjer, 2013). After comparison between the current vocalization and the stored model, the motor program for speech or song may be adjusted so that the vocalization can develop to better match the stored model (Achiro and Bottjer, 2013). As a result, both species are able to produce vocalizations that match encoded templates. The third phase occurs around the first birthday in humans and 90 dph in zebra finches. By ten months, humans can produce language-specific speech and around their first birthday, they are producing their first words. In parallel, at 90 dph, zebra finches are able to develop their crystallized adult song, which consists of stereotyped motifs that match the stored tutor template (Doupe and Kuhl, 1999, Figure 2). This song remains fairly stable throughout the rest of the finch's life.

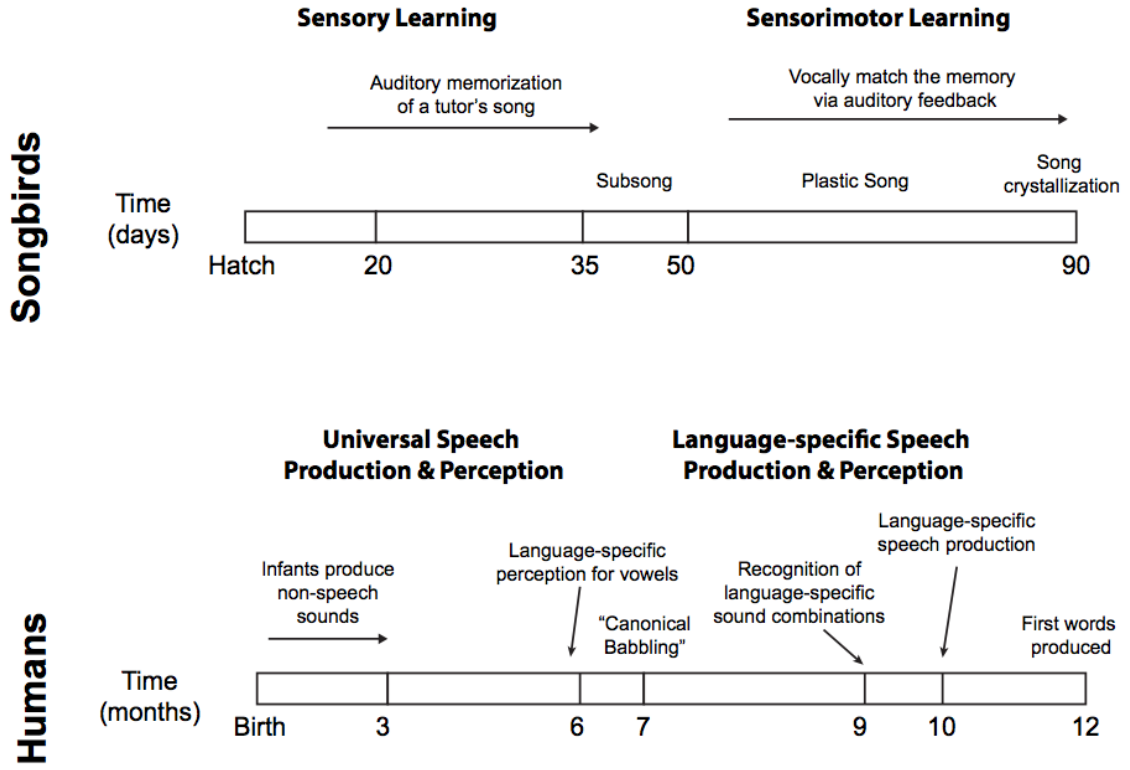

Figure 1: Parallels between human speech and zebra finch song development. Figure from Olson et al. (*in review*), adapted from Doupe and Kuhl, 1999.

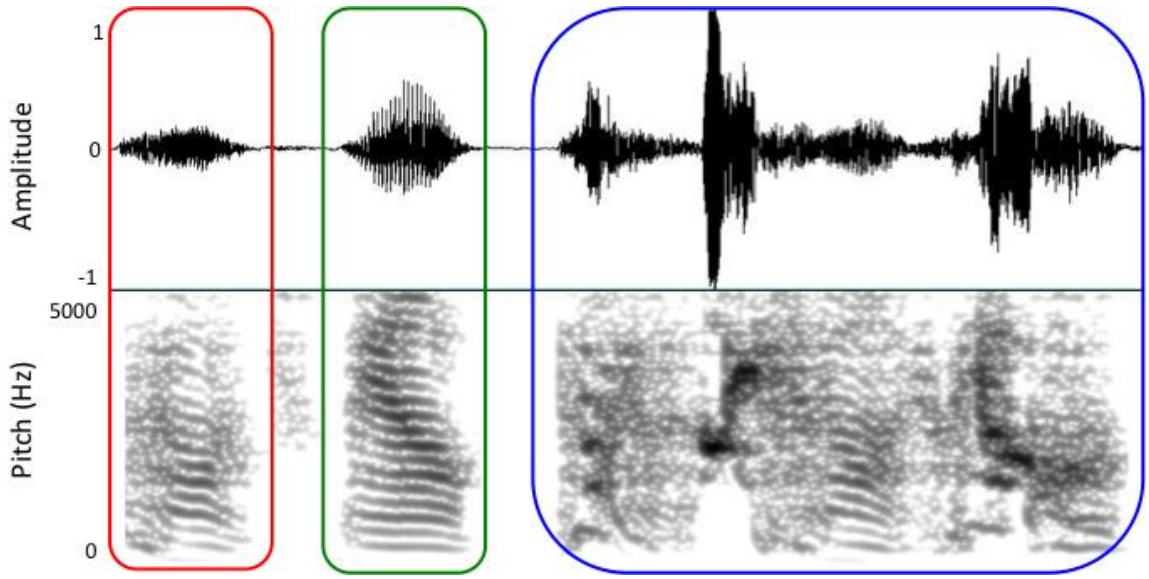

Figure 2: Example motif from the crystalized song of an adult zebra finch. Amplitude of sound is depicted on the top; pitch (Hz) is depicted on the bottom. The outlines define the different sub-syllables of the motif. This motif is repeated multiple times in a song bout.

Not only are there developmental similarities, but there are also distinct neuroanatomical similarities between humans and zebra finches for speech and song production and perception, respectively. In humans, speech production is associated with a region in the frontal lobe known as Broca's area, and language comprehension pertains to a region known as Wernicke's area, located in the temporal lobe. Parallel regions in the zebra finch brain are the NCM, which encodes the memory for tutor song, and the HVC, which controls the brain motor pathway that directs song production (Hahnloser, Kozhevnikov, and Fee, 2002; Leonardo and Fee, 2005; Bolhuis et al., 2012; Bolhuis and Moorman, 2015, Figure 3).

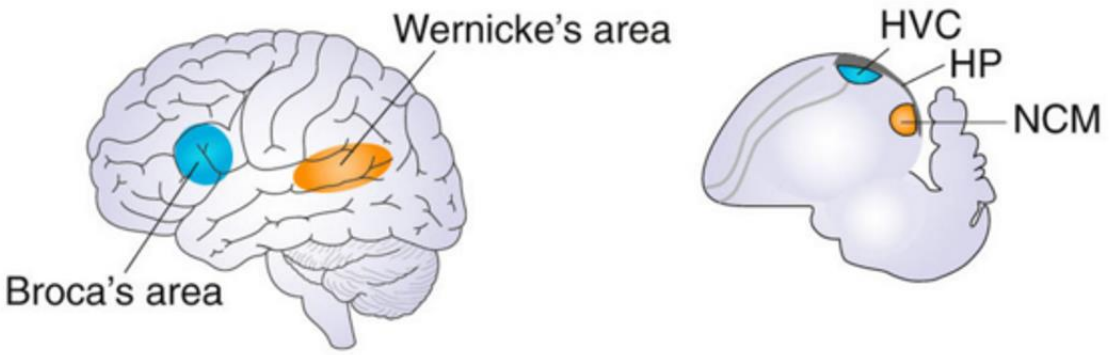

Figure 3: Human and zebra finch regions of vocal processing and production. Broca's area (blue) in the human brain (left) is associated with speech production, which is analogous to HVC (blue) in the zebra finch brain (right). Wernicke's area (orange) in the human brain (left) is associated with speech comprehension, which is analogous to NCM (orange) in the zebra finch brain (right). HP on zebra finch brain diagram labels the hippocampus.

In both species, production and perception of speech and song, respectively, are localized and dissociated to distinct regions of the brain (Gobes and Bolhuis, 2007). Furthermore, lateralized processing and production in the above regions occur in both species. Monolingual humans exhibit left-lateralized activation in language-related brain regions during language-related tasks; this left-dominant activation develops with

language experience and proficiency (Conboy and Mills, 2006; Dehaene-Lambertz et al., 2002; Friederici, A.D., 2011; Olson et al., *in review*). Right-lateralized activation appears to be detrimental to language development, as right-lateralized language processing underlies considerable speech disorders (Eyler et al., 2012; deGuibert et al., 2011; Berl et al., 2014; Oertel et al., 2010; Sommer et al., 2011; Johnson et al., 2013; Olson et al., *in review*). In parallel, left-lateralized activation in the NCM of juvenile male finches is associated with tutor song similarity; the greater the similarity between the bird's current song and its tutor's song, the greater left-lateralized neuronal activation in NCM in response to the tutor song (Moorman et al., 2012). Therefore, it appears that lateralized processing is an evolutionarily advantageous trait underlying both human language and zebra finch song development. Although language-related brain activation in monolingual humans is generally agreed to be left-lateralized, the degree of lateralization in brain activation to second language in bilingual humans and the extent of overlap in activation to the first and second language is still under considerable debate (Mingawa-Kawai et al., 2011). Current evidence suggests that greater proficiency with the second language and exposure to the second language earlier in development results in greater left lateralized activation and a higher degree of overlap in activation with the first language (Perani et al., 1996; Kim et al., 1997; Perani et al., 1998; Raboyeau et al., 2010). In previous work, we used sequentially tutored zebra finches to model the sequential learning of multiple languages exhibited by bilingual humans (Olson et al., *in revision*). We exposed juvenile zebra finches to one tutor early in development (between birth and 30 dph) and to a different tutor, with a dissimilar song to the first tutor, later in development (between 60 and 90 dph). At day 93, when the zebra finches reached

adulthood, finches were exposed to either their first tutor or second tutor song in order to activate *Zenk* (an acronym for *zif268*, *egr-1*, NGFI-A and *krox-24),* an immediate early gene that is quickly transcribed in some brain regions in response to neuronal activity. After immunocytochemical processing of the tissue, we looked at neuronal activation in the NCM.

We found that lateralized neuronal activation in NCM was related to song similarity to the first or second tutor. For adult birds exposed to their first tutor, we found a significant correlation between right-dominant neuronal activation in the NCM in response to first tutor song and similarity to the first tutor song (Figure 4, top left). This suggests that traces of the neural representation for the first tutor song can persist in the right hemispheric NCM, dependent on maintenance of the first tutor song, even after exposure to a different second tutor. For adult birds exposed to their second tutor song, we found a significant correlation between left-dominant neuronal activation in the NCM in response to second tutor song and similarity to the second tutor song (Figure 4, bottom right). This indicates that traces of the neural representation for the second tutor song are acquired by the left hemispheric NCM and are also dependent on the degree of second tutor song acquisition later in development.

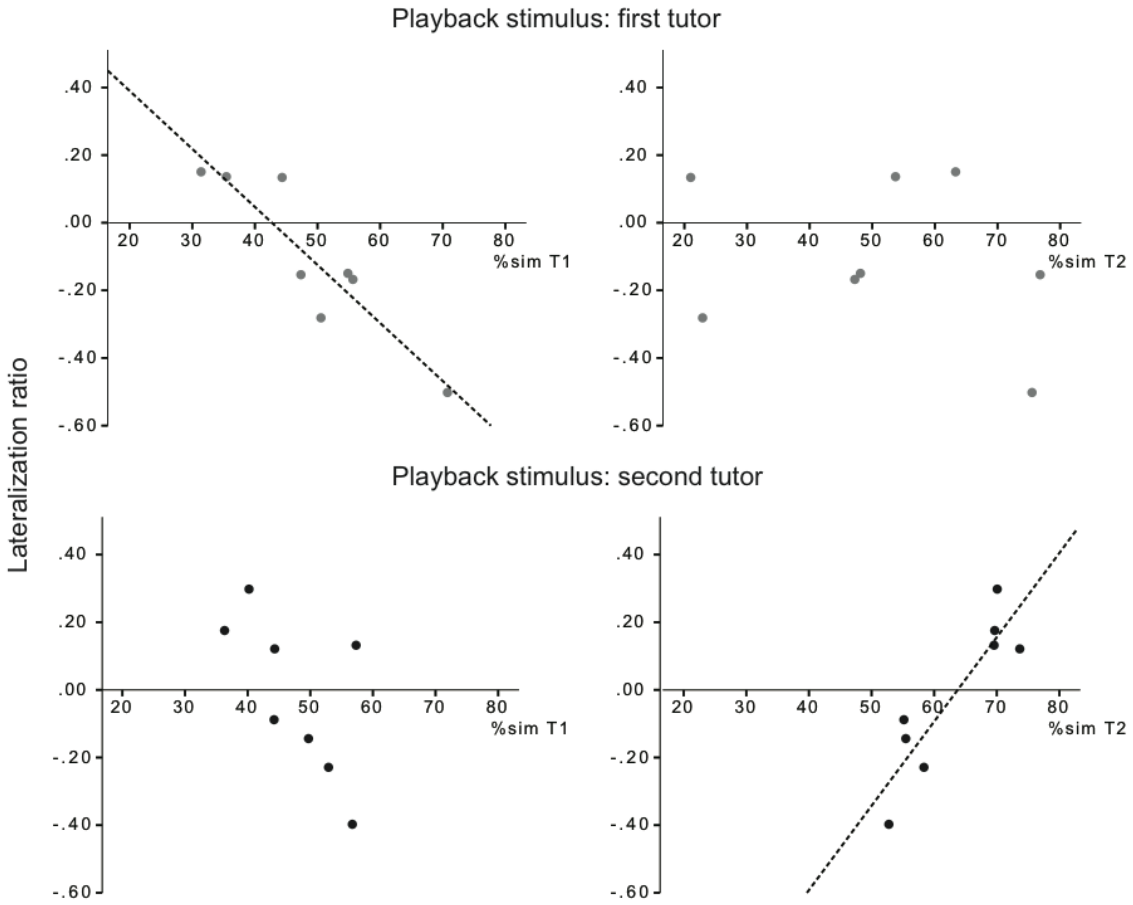

Figure 4: Similarity to first and second tutor song exhibit correlations with left and right-lateralized activation in the NCM, taken from Olson et al. (*in revision*). Significant right-lateralized neuronal activation in NCM to first tutor song correlated with similarity to first tutor; significant left-lateralized neuronal activation in NCM to second tutor song correlated with similarity to second tutor song. Parallel correlations show no significant relationships. Lateralization ratio calculated as [L-R]/[L+R]; positive values indicate right-dominant expression, negative values indicate left-dominant expression.

This study suggests that the zebra finch brain is able to maintain representations for song to which the zebra finch was exposed to for a limited duration early in development, while also acquiring the neural representation for a novel, different song later in development. The reactivation of old and new neural representations in adulthood depends on proficiency, which parallels human studies demonstrating proficiency-dependent second language lateralization. Furthermore, these results suggest dual

processing in the left and right hemispheric NCM; the right NCM may be more resistant to novel auditory input and is better able to maintain an early-stored representation for tutor song, while the left NCM may exhibit a greater degree of plasticity and is better able to respond and adapt to a novel auditory environment and encode the representation for a novel tutor song.

Although there are several arguments to be considered in explaining lateralization for first and second tutor song representations, including the rate of song learning and the degree of song salience (see Olson et al. (*in review*) for details); here, we focus on this plasticity-based theory underlying the left-dominant response to a novel auditory environment.

Neurogenesis in the Zebra Finch Brain

The addition of new neurons in adulthood challenges previously instituted critical periods for brain plasticity and strengthens the idea that the brain is a dynamic organ that remains highly responsive to its environment throughout the lifetime. New neurons in the zebra finch brain are born in the telencephalic ventricular zone (VZ) and migrate broadly to a variety of brain regions involved in song perception and production (Alvarez-Buylla and Nottebohm, 1988; Barnea and Pravosudov, 2011, Figure 5). New neurons are added to several regions, including the HVC and NCM; however, the level of recruitment and the survival of new neurons vary depending on the region and external environmental factors that regulate adult neurogenesis (Alvarez-Buylla and Nottebohm, 1988; Barnea and Pravosudov, 2011).

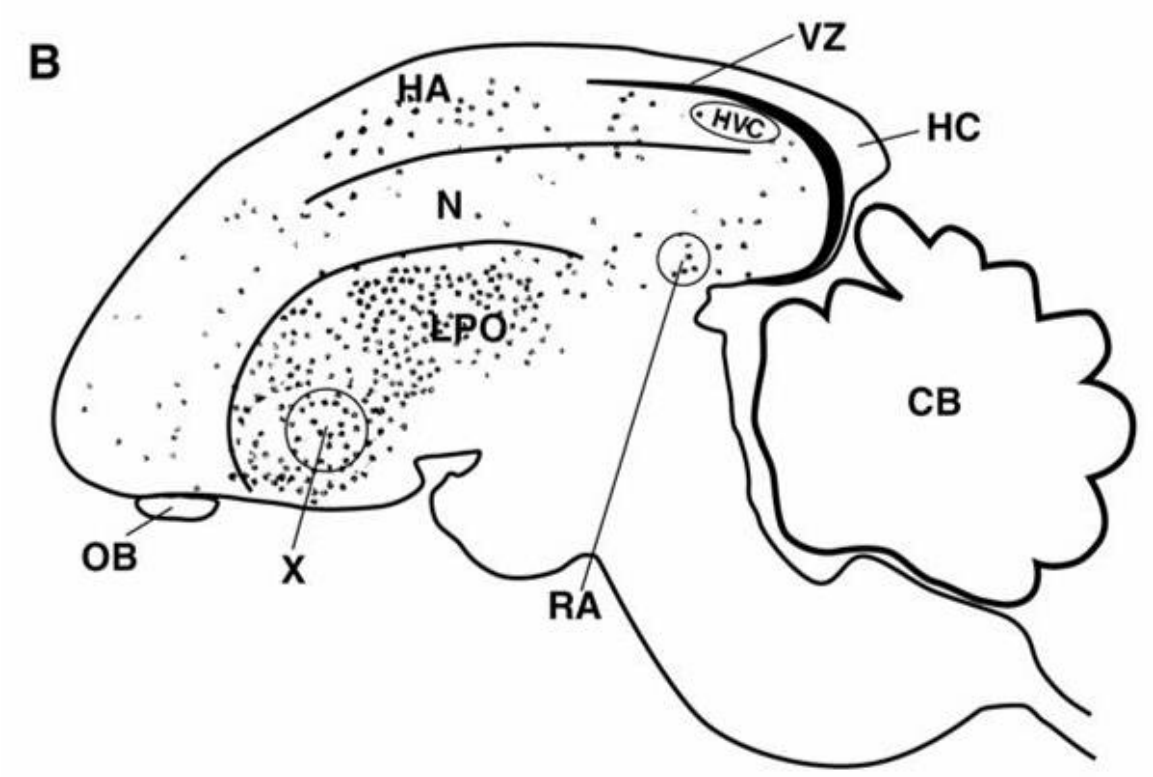

Figure 5: Map of neurogenesis in the adult zebra finch brain, taken from Barnea & Pravosudov (2011). New neurons are added to several sensory and motor regions, including HVC, the song premotor area, and N, the primary auditory region containing Field L and NCM. More dense clusters indicate higher levels of new neuron incorporation.

Neurogenesis in the HVC

Neurogenesis in HVC appears to underlie song maintenance, rather than plasticity, as new neuron addition in HVC continues through adulthood even though the bird's adult song is stable and remains relatively constant. HVC consists of three different neuronal types: HVC interneurons, HVC neurons projecting to the robust nucleus of the archistriatum (RA), which control the muscles in the syrinx, and HVC neurons projecting to Area X, a region in the anterior forebrain pathway (analogous to the mammalian basal ganglia) (Spiro et al., 1999; Gale and Perkel, 2010). Adult neurogenesis in the HVC is specific to HVC interneurons and HVC-RA neurons, as the peak addition of HVC-X

neurons occurs prenatally and they are not added during adulthood (Alvarez-Buylla, et al., 1988). New HVC neurons do not appear to function to replace older, dying neurons; the volume of the HVC appears to slightly increase over time and the diameter of new neurons is smaller, suggesting that new HVC neurons serve to add to the volume of the HVC rather than replace and maintain a constant volume (Walton, Pariser, and Nottebohm, 2012). Recent studies suggest that new HVC neurons in the zebra finch contribute to song stability, rather than the contribution to variability observed in other songbird species (Alvarez-Buylla et al., 1988; Kirn et al., 1994; Tramontin & Brenowitz, 1999; Scott et al., 2000). Between 90 dph and 11 years of age, the number of HVC-RA neurons doubles, yet the song remains fairly stable (Walton et al., 2012). New neurons added to the HVC of adult zebra finches during a period of deprived auditory feedback appear to maintain crystallized adult song. Greater new neuron recruitment during deafening correlates positively with song similarity (between the bird's own song and tutor song) and song accuracy (regarding syllabic maintenance), whereas decreases in song similarity and accuracy are related to reduced new neuron recruitment (Pytte et al., 2011; Pytte et al., 2012). Social complexity also appears to be involved with neurogenesis in the HVC; zebra finches raised in a more complex environment, such as in cages with a multitude of other male zebra finches, recruit more HVC-RA neurons than zebra finches raised in isolation (Adar et al., 2008). This suggests that HVC-RA neurons may function to maintain stable song in an environment in which each individual bird's song must remain distinct and stable so that birds are better able to navigate their environment socially. The results also indicate that HVC neurons may contribute to perception of conspecific songs, however, further research must be conducted in order to explore this

theory (Adar et al., 2008). The current evidence suggests that HVC-RA neurons are added throughout adulthood with the purpose of maintaining crystallized adult song.

Neurogenesis in caudomedial Nidopallium (NCM)

While HVC may recruit new neurons for maintenance, the NCM appears to recruit new neurons not only for maintenance, but also for plasticity and flexibility. New neuron recruitment in the zebra finch brain is related to increased social complexity. When zebra finches are housed in environments with other zebra finches as opposed to housed in isolation, more new neurons are recruited to several regions, including area NC (which includes the NCM) and these new neurons turnover faster (Lipkind et al., 2002; Adar et al., 2008; Barnea et al., 2006). This results in replacement of new neurons with newer neurons, suggesting rapid responses to the complex environment (Lipkind et al., 2002; Adar et al., 2008; Barnea et al., 2006). These studies suggest that zebra finches in environments with many conspecific songs exhibit increased neuronal plasticity, as evidenced by increased new neuron addition and faster neuron turnover, in order to be able to navigate a complex auditory and social environment. Potentially, NCM requires new neurons to promote enhanced auditory perception and categorization of conspecific songs, resulting in short-term neuronal representations of cage-mate songs in NCM. Use of the region is also a key regulator of neurogenesis in the NCM. Deafened birds recruit significantly fewer new NCM neurons than hearing birds, which suggest a necessary role for enhanced auditory input in increasing new neuron recruitment in the NCM (Pytte et al., 2010). However, there may be multiple functions for new neuron recruitment in NCM. Although deafened birds recruited significantly fewer new neurons than hearing

birds, these birds still recruited a substantial population of new neurons, suggesting that a baseline level of neurons are added to maintain the function of the region, regardless of deprived input. Furthermore, there appear to be regional differences in the addition of new neurons that may differentially regulate the nature of the stored neural representation of song memory. The medial and dorsal NCM were more disrupted by deafening than lateral and ventral areas. This indicates that these areas may be involved in encoding neural representations for conspecific songs, as deafening to conspecific song may result in reduced new neuron recruitment in these regions that allow for neuronal representation and categorization of cage-mate songs (Pytte et al., 2010). The lateral and ventral areas may be potential storage areas of tutor song; more new neurons were possibly added to these regions to strengthen the existing representation of tutor song (Pytte et al., 2010). This shift towards strengthening the existing memory may be a result of lack of conspecific input. Furthermore, left-lateralized new neuron addition in adulthood is positively correlated with fidelity of tutor song imitation, which suggests that new neurons may allow for the stabilization of the tutor song template in the left hemisphere throughout development, resulting in more successful tutor song imitation (Tsoi et al., 2014). Potentially, left dominant addition of new neurons in the NCM underlies the ability of the left NCM to better adjust to the novel auditory environment posed by exposure to a novel tutor.

Neurogenesis in the Context of Sequential Tutoring and Learning of Multiple Song Models

In this thesis, we will examine differences in new neuron addition and preferential recruitment of new neurons in tutor song representations in NCM and HVC between

birds and determine how the behavioral and neural data fit together to explain potential differences in successful acquisition of the second tutor song. Furthermore, we will examine whether new neurons facilitate enhanced auditory and motor plasticity, even though the hypothesized critical period for song learning has passed. Previous work implicates new neuron recruitment in the HVC and NCM in the stability of song production and the stability and flexibility of neural representations for tutor song and possibly conspecific song. Thus the role of new neurons in response to a novel, second tutor song later in development should be investigated. We hypothesize that greater left-lateralized new neuron recruitment in the left NCM will underlie more successful acquisition of the second tutor song, and greater new neuron recruitment in the HVC will underlie greater maintenance of first tutor song, even after exposure to a second tutor song.

2. EXPERIMENTAL PROCEDURES

2.1 Animals and rearing protocol

Nine male zebra finches (*Taeniopygia guttata*) were reared in the animal facility at Wellesley College with controlled auditory and social exposure to adult song tutors. Birds were maintained on a 16:8 light: dark cycle, lights on at 10:00 am. All birds were reared in breeding cages with their father, mother, and siblings, and each clutch was housed in acoustically isolated single-clutch holding cages until 33 dph (Figure 6). At 33 dph, juvenile males were transferred into individual, sound attenuating chambers (Figure 6). At 35 dph, birds received injections three times per day for four consecutive days of BrdU (5-Bromo-2'-deoxyuridine, 10 mg/ml, Figure 6). At 55 dph, juvenile males were paired with a second tutor, an adult male zebra finch that was not the biological father, with whom they were housed for the next 10 days (Figure 6). At 65 dph, birds were separated from their second tutor (Figure 6). At 92 dph, birds were transferred into acoustically isolated chambers set-up for playback of sound stimuli (Figure 6). Experimental procedures were in accordance with US law and approved by the Institutional Animal Care and Use Committee of Wellesley College (IACUC #1106 and #1405).

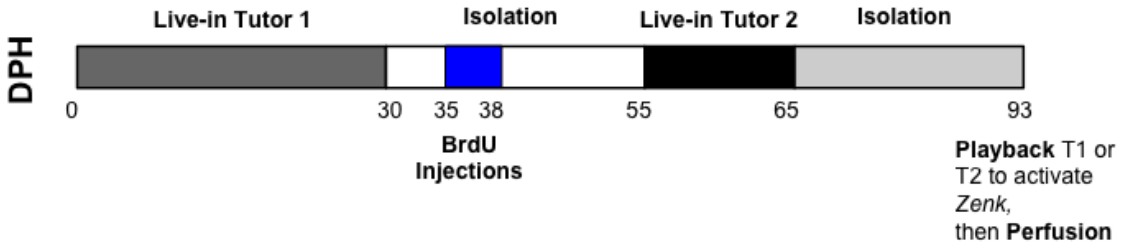

Figure 6: Experimental timeline. Male zebra finches lived with their first tutor, their father between 0 and 30 days post hatch. At day 30, male zebra finches were isolated from their father, mother and siblings and put in separate cages. Between days 35 and 38, BrdU was injected intramuscularly 3 times per day (7:00 am, 3:00 pm, and 11:00 pm) for 4 consecutive days. Once the birds turned 55 days old, a novel, second tutor was placed

in their cage as a live-in tutor for 10 days. At day 65, the bird was separated from the second tutor and re-isolated until day 93. At day 93, animals were exposed to either their first or second tutor song to elicit Zenk expression and then perfused.

2.2 Behavioral analysis

Sound data was collected continuously from each experimental bird, beginning at time of separation, when birds were placed in the soundproof chambers, through time of sacrifice, and included data from birds individually and together with the first or second tutor. Vocalizations were monitored and digitally recorded with directional microphones (Shure SM93, Shure Incorporated, Niles, IL, USA) using custom written software. Songs used for similarity analysis were taken from days that the birds were housed individually between tutoring experiences (at 54 dph) and right before the behavioral experiments (at 91 dph). Four comparisons were made for each bird: similarity to first tutor song post-exposure at 54 dph, similarity to second tutor song pre-exposure at 54 dph, similarity to first tutor song at 91 dph, and similarity to second tutor song at 91 dph. Second tutor pairings were determined based on analysis of similarity scores (see below), and tutors were selected to optimize learning based on low similarity scores between second tutor song and first tutor song.

We used "Sound Analysis Pro (2011)" (Tchernichovski and Mitra, 2004) to measure the overall similarity of the bird's own song (BOS) to either of its tutors' songs. The "percentage similarity" that is calculated by Sound Analysis Pro, is an objective quantification of the fidelity of song imitation based on multiple acoustic parameters: pitch, Wiener entropy, frequency modulation (FM), and spectral continuity (Tchernichovski et al., 2000). To compare the song of a juvenile zebra finch to its tutors' songs, we identified the most frequently repeated single motif at 54 dph and 91 dph (days

of song analysis) and randomly selected 10 examples of this motif between 3 pm and 6 pm. Using paired comparisons between tutor and tutee, we compared 10 single motifs from the tutee song to 10 motifs from each tutor song (excluding introductory notes) to calculate the average percentage similarity. The number of syllables in each motif was found by importing the motif into PRAAT (Phonetic Sciences, Amsterdam, The Netherlands) and counting the number of syllables in the time-versus-amplitude spectrum. On SAP, motifs of tutor song and tutee song were input in pairs and decibel level was set to the maximum level (See Appendix, Figures 25 & 26) The amplitude of each song file was adjusted until the number of syllables presented on the time-versus-amplitude spectrum matched the number of syllables found in PRAAT. The average percentage similarity for each pair of tutor motifs and tutee motifs and syllable-by-syllable percent comparisons between the two song files were also recorded (See Appendix, Figure 27). One bird was eliminated from song analysis due to low quality recordings at 54 dph, resulting in pairing with a second tutor that was too similar to be regarded as novel, therefore violating the rules for second tutor pairing in the experiment.

2.3 Procedures for playback experiments

One day prior to stimulus exposure at 92 dph, birds were put in a cage measuring 40 x 35 x 35 cm and placed within a soundproof chamber equipped with a microphone and speaker, with water and food available *ad libitum.*

On the day of the experiment, the lights were manually turned on for the duration of stimulus exposure. Stimulus presentation started between 10:00 AM and 11:00 AM and lasted 30 minutes. The birds were sacrificed 30 minutes after the end of the last stimulus presentation. The birds were kept in darkness during the 30 minutes

post-stimulus period to stop them from vocalizing, and thereby to prevent their own vocalizations from evoking molecular neuronal activation. The stimulus consisted of a recording of the song of their first tutor (father, TUT1), or a recording of the song of the second tutor (TUT2). Birds were divided over the two groups (TUT1 exposure or TUT2 exposure) semi-randomly as to maximize diversity in each group. That is, we made sure each group represented a range of learning outcomes with respect to retention of the song learned first or the degree of switching over to the second song (Olson et al., *in review*).

Each 30 minute stimulus consisted of one-minute loops in which 15 seconds of sound was followed by 45 seconds of silence. The stimulus songs were broadcast through a speaker and Windows Media Player controlled the sound pressure level at 65 dB mean SPL at 30 cm through a speaker. Sound recordings were made throughout the experiment to ensure that birds were awake during stimulus presentation, and to monitor vocal behavior during stimulus exposure.

2.4 Tissue collection

One hour after stimulus onset, the experimental subjects were anesthetized with 0.03 mL Natriumpentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) and subsequently perfused with phosphate buffer (PB, pH 7.4) containing 0.2% heparin, followed by fixation with 2% paraformaldehyde. Whole brains were dissected out, separated by hemisphere and post-fixed at 4°C in 2% paraformaldehyde in PB overnight. Parasaggital sections (50 μm) were made on a Leica vibratome (Leica Biosystems, Buffalo Grove, Illinois) and stored in cryoprotectant at -18°C.

Description of Methods in Section 2.1 to 2.4 adapted from Olson et al. (in review) and

Chirathivat et al. (2015).

2.5 Immunocytochemistry

The immunocytochemistry protocol was adapted from Kee et al. (2007). Six sections from each hemisphere were collected for NCM; medial sections were between 0 and 600 micrometers from the midline and lateral sections were greater than 600 micrometers from the midline. Three sections from each hemisphere were collected for the HVC; sections were between 1700 and 2200 micrometers from the midline and about 100 micrometers apart from each other. Sections were rinsed three times in PB (5 min. per rinse) and incubated in HCl (1 N) for 30 minutes at 45° C. Sections were rinsed three times in PBS (5 min. per rinse) to neutralize acid. Sections were incubated in BrdU (1:500), egr-1 (1:1000) and Hu (1:100) in blocking solution (0.1 M PBS, 0.3% Triton-X, and 2% Normal Goat Serum) for 48 hours at 4° C on an orbital shaker. Sections were rinsed three times in PBS (5 min. per rinse) and then incubated in CY-3 (1:500), CY-5 (1:500), and CY-2 (1:500) in blocking solution (0.1 M PBS, 0.3% Triton-X) for 2 hours in the dark at room temperature. Sections were rinsed three times in PBS (5 min. per rinse) and then placed briefly in water. Sections were mounted on slides; Fluoromount-G was applied to sections and slides were coverslipped. All procedures were performed cold (4° C) unless otherwise specified. Control sections were also stained, with primary or secondary antibody omitted (see Appendix, Figure 20).

2.6 Microscopy

Images were taken on Leica Microsystems TCS SP5 microscope using Leica Microsystems LAS AF software (Leica Microsystems GmbH, Wetzlar, Germany). The

objective was set to 20 x 0.80 DRY UV and images were taken between frames in sequential scan mode. The shutter speed was set to 400 Hz and the dimensions of the image were set to 512 x 512 pixels, or 775 x 775 micrometers. Scan field rotation was set to 0.0° and zoom was at 1.00. To image BrdU, HeNe 543 was set to a power of 41 %. The smart gain was set to 811.0V, smart offset at -2.2%; the excitation range was between 480 and 540 nm and the detection range was set to 550 and 640 nm. The line average was 1 and the frame average was 4. To image Zenk, HeNe 633 was set to a power of 41%; settings for Leica/ALEXA 647 found on system were used. The smart gain was set to 779.0 V, smart offset at -18.0%; the excitation range was between 630 and 780 nm and the detection range was between 680 and 720 nm. The line average was 1 and the frame average was 4. To image Hu, we used the settings for Leica GFP (Alexa 488) found on the system. The Argon laser was set to 20-30% power and the laser power was set to 15%. The smart gain was set to 811.0 V, smart offset at -8.0%; the excitation range was between 480 and 600 nm and the detection range was between 500 and 600 nm. The line average was 1 and the frame average was 4. Smart gain and offset were adjusted if necessary, but rarely. For NCM, 14 successive slices at a width of 1.05 micrometer were taken in 15 steps for a total depth of 14.70 micrometers. Fro HVC, 15 successive slices at a width of 1.05 micrometers were taken in 16 steps for a total depth of 15.75 micrometers.

2.7 Image Analysis

The 15-section z-stacks collected for NCM were collapsed and snapshots of Zenk collapsed stack was taken. Minimum signal intensity was slightly reduced in order to

reduce background. Snapshot was input in ImageJ 2.0 (Schindelin et al., 2015); the image was converted from RGB-color to a 16-bit image. The threshold was then adjusted so that the bright Zenk labeled-cells were included in analysis (to verify that the correct cells were included, the tif file was opened next to the ImageJ file). A median pixel radius of of 2.0 was applied and then watershed segmentation was conducted to separate objects that were close together. The image was then inverted from white objects on a black background to black objects on a white background. Finally, the analyze particles program was run and a cell count was obtained. This count was then divided by the area of microscope capture (775 x 775 micrometers) to obtain an area density.

We first visually confirmed that there was 100% overlap in Zenk+/Hu+ labeled cells so that we could reduce the intensity of the Hu stain when counting the Zenk+/BrdU+ labeled cells. In the Leica software, the brightness of channel 3 (Hu stain) was reduced to -100 from baseline (0) so only channels 1 (BrdU) and 2 (Zenk) were visible in the stacks. This allowed for better view of BrdU+/Zenk+ labeled cells, as the Hu is a very bright and occluding stain. The 15 section z-stacks for NCM were exported and randomly coded and then one-by-one, each stack was input in FIJI (Fiji Is Just Image J, Schidelin et al., 2012). Images were converted to a stack, allowing the viewer to scroll through each section of the stack in depth order. BrdU+/Zenk+ labeled cells were manually identified throughout the stack and then counted to obtain a measure of BrdU+/Zenk+ labeled cells for each section. This count was then divided by the volume of microscope capture (775 x 775 x 14.70 micrometers) to obtain a volume density.

In the Leica software, the brightness of channel 2 (Zenk) was reduced to -100 from baseline (0) so that only channels 1 (BrdU) and 3 (Hu) were visible. This allowed

for better view of BrdU+/Hu+ labeled cells as Zenk is a dark-colored and occluding stain. The 15 section z-stacks for NCM were exported and randomly coded and then one-by-one, each stack was input in FIJI (Schindelin et al., 2012). Images were converted to a stack to allow the viewer to scroll through each section of the stack in depth order. BrdU+/Hu+ labeled cells were manually identified throughout the stack and then counted to obtain a measure of BrdU+/Hu+ labeled cells for each section. This count was then divided by the volume of microscope capture (775 x 775 x 14.70 micrometers) to obtain a volume density.

The 16 sections z-stacks for HVC were exported and then randomly coded. Coded stacks were then input in FIJI (Schindelin et al., 2012) one-by-one and images of each stack were converted to a stack to allow the viewer to scroll through each section of the stack in depth order. BrdU+/Hu+ labeled cells were manually identified throughout the stack and then counted to obtain a measure of BrdU+/Hu+ labeled cells for each section. One of the images in the stack was opened in ImageJ; the area of HVC was traced and then a measure of the area within the boundaries of the tracing was obtained. The BrdU+/Hu+ labeled cell count was then divided by the volume of the microscope capture (775 x 775 x area obtained in ImageJ micrometers) to obtain a volume density.

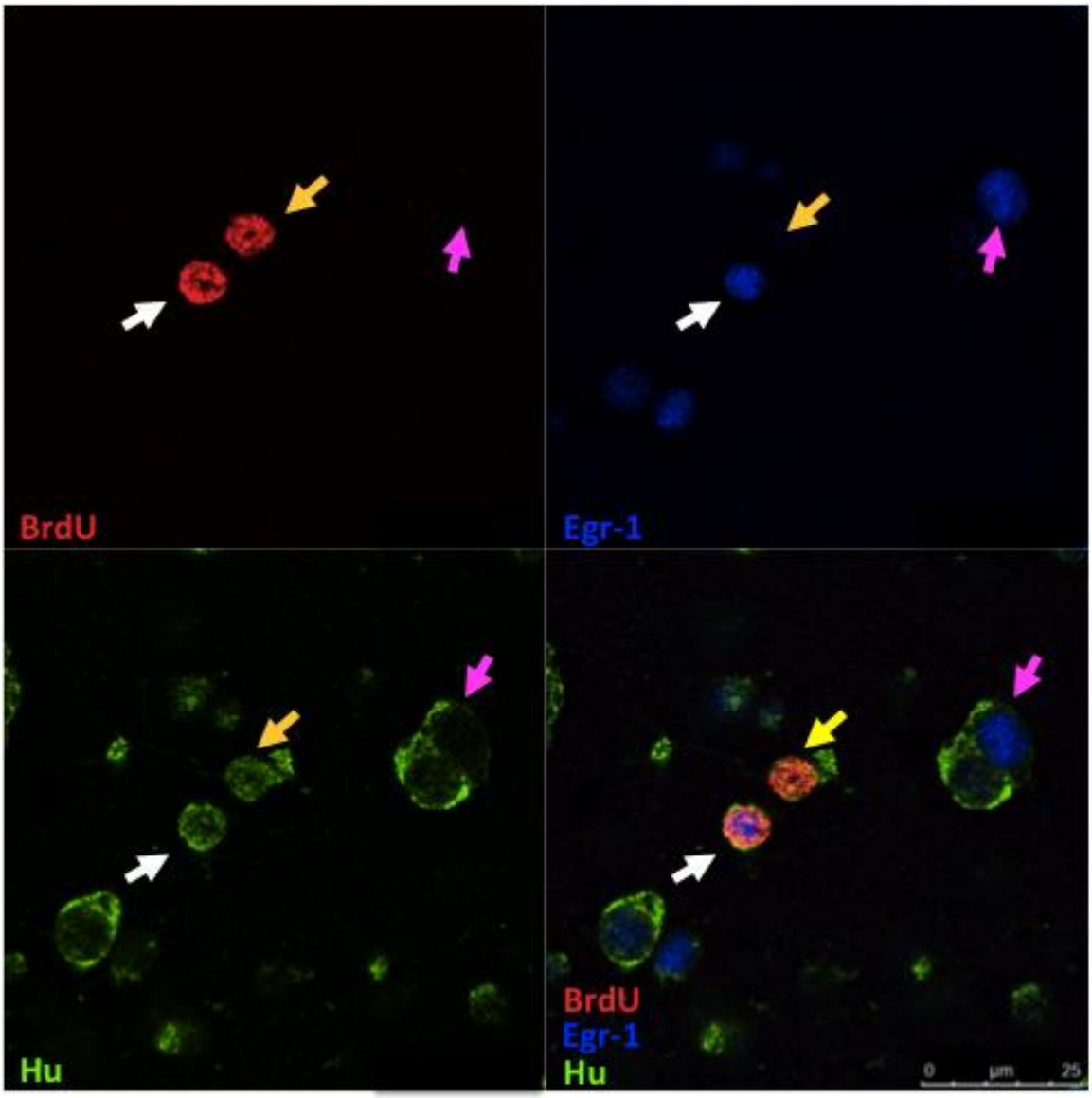

Figure 7: Different types of cells in NCM. Red-stained cells indicate BrdU+ labeled cells, blue-stained cells indicate Zenk+ labeled cells, and green-stained cells indicate Hu+ labeled cells. BrdU+/Hu+ labeled cell indicates new neuron (yellow arrow). BrdU+/Hu+/Zenk+ labeled cell indicates new neuron recruited for tutor song template (white arrow). Hu+/Zenk+ labeled cell indicates mature neuron recruited for tutor song template (pink arrow). Image taken at 40x magnification.

2.8 Statistical analysis

To test whether birds had learned from their first or second tutor, we used paired t-tests. We conducted repeated-measures analysis of variance (ANOVA) to examine the effects of playback stimulus on the Zenk response in the different brain regions and

experimental groups. We conducted paired-samples t-test to determine if there was a difference in new neuron addition in the left and right HVC. We also conducted a bivariate correlation to determine the relationship between new neuron addition in the two hemispheres of HVC. We conducted a paired sample t-test to determine if there was a difference between new neuron density and new neuron recruitment in the left and right NCM. We conducted correlations to examine the effects of new neuron density and new neuron recruitment in first and second tutor song templates on maintenance of first tutor song and acquisition of second tutor song in both NCM and HVC. We also ran correlations of the lateralization ratio of Zenk density, new neuron density, and new neurons recruited for tutor song density against learning scores to the first tutor and the second tutor. Data were analyzed using SPSS 22.0.0 (IBM Corporation).

3. RESULTS

3.1 Song Learning

We followed a rearing protocol similar to that developed by Yazaki-Sugiyama and Mooney (2004), raising male zebra finches with a first tutor and subsequently with a second tutor, which resulted in successful extension of the sensory acquisition phase beyond 55 days post hatching (dph) (Olson et al., *in review*). At 54 dph, or right before the birds were paired with their second tutor, song analysis showed that birds had started to copy elements of their first tutor song, resulting in significantly higher song similarity to the first tutor at 54 dph compared to similarity with the second tutor song at 54 dph ($t(7) = -.170$, $p = 0.033$; Figure 8, visually seen in Figure 10). These results match those found in Olson et al. (*in review*), where bird's song at 54 dph was significantly more similar to their first tutor than the novel, second tutor. The similarity score between the tutee and the second tutor at 54 dph can be used as a baseline level of similarity because the tutee has not yet been exposed to the second tutor song at 54 dph.

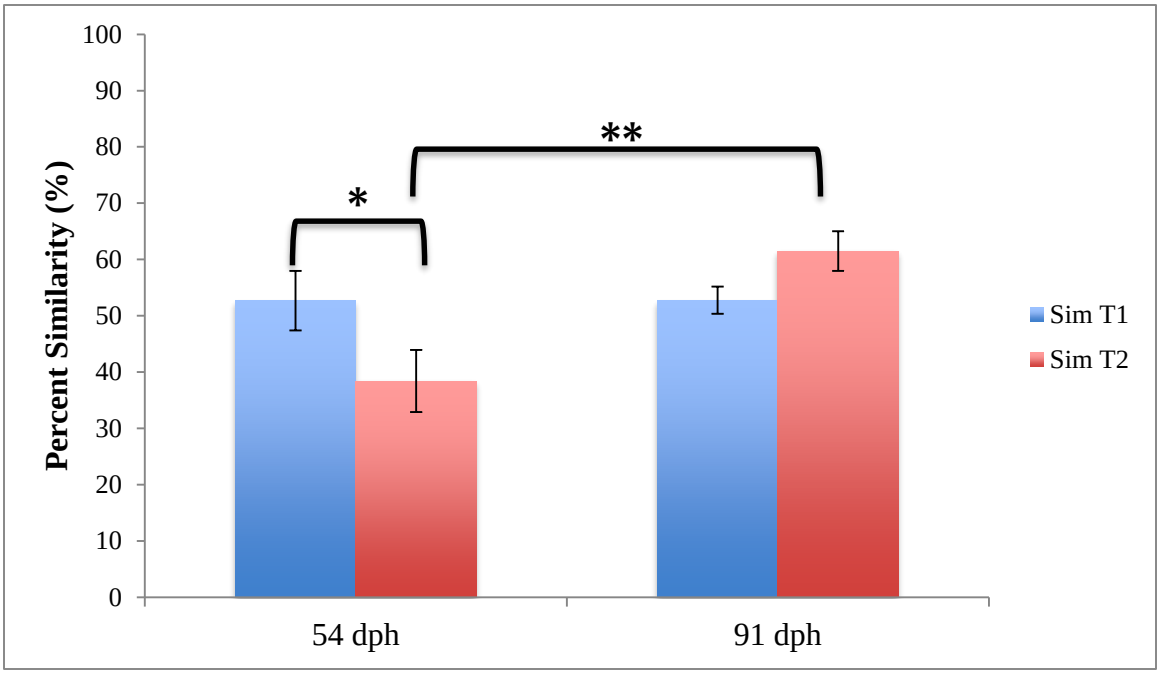

Figure 8: Mean changes in similarity to Tutor 1 song and Tutor 2 song over time. Blue bars indicate mean similarity (±SEM) to Tutor 1 song. Red bars indicate mean (±SEM) similarity to Tutor 2 song. Single asterisk (*) indicates significant difference between similarity to Tutor 1 song and Tutor 2 song at 54 dph (n = 8, p = 0.032. Double asterisk (**) indicates significant difference between similarity to Tutor 2 song at 54 dph and 91 dph (n = 9, p = 0.002). Mean at 54 dph and 91 dph to Tutor 2 includes all 9 birds; mean at 54 dph and 91 dph to Tutor 1 includes 8 birds. This analysis excluded the 1 bird that was paired with a second tutor who could not be regarded as novel based on the high similarity between the second tutor song at the bird's song at 54 dph.

After 10 days of exposure to their second tutor, the adult birds (at 91 dph) exhibited copying from the second tutor, as reflected by significant increases in similarity scores with the second tutor song between 54 dph and 91dph ($t(7) = -4.878$, $p = 0.002$; Figure 8 and Figure 9, visually seen in Figure 10). Again, these behavioral results match those observed in Olson et al. (*in review*), in which we found that birds significantly learned from their second tutor between 54 dph and 91 dph and the bird's song similarity to the first tutor did not change over the course of development past 54 dph. We also tracked the change in Tutor 1 song similarity between 54 dph (after exposure to Tutor 2) and 91 dph (after exposure to both Tutor 2 and Tutor 1) (Figure 8 & Figure 9). We found

no significant difference in similarity to Tutor 1 song as a result of the period of exposure to Tutor 2 (Figure 9).

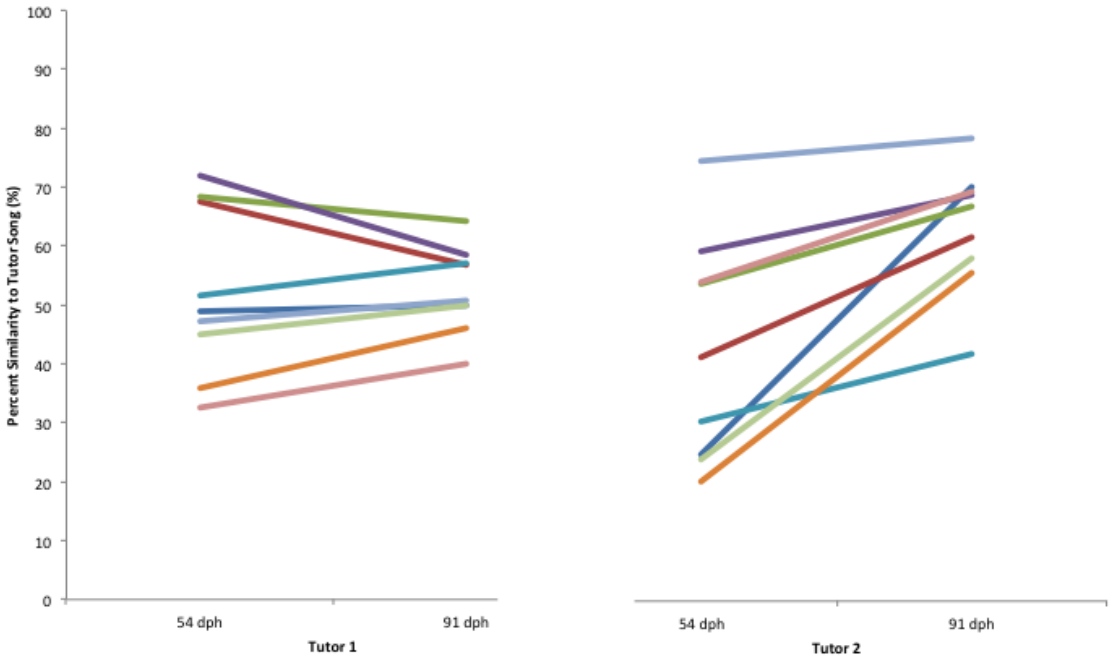

Figure 9: Change in percent similarity to Tutor 1 (left) song and Tutor 2 (right) song over development. Each colored line indicates an individual zebra finch.

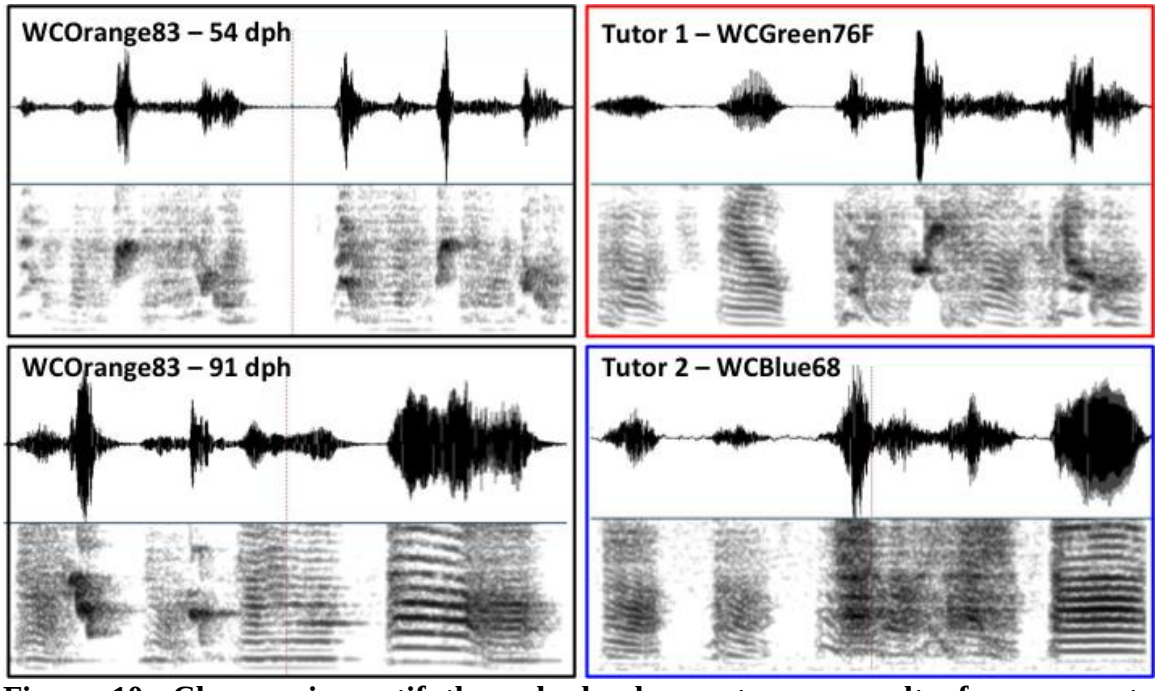

Figure 10: Changes in motif through development as a result of exposure to

different tutors can be observed in the example above. Top left motif is from WCOrange83 at 54 dph, after tutoring by Tutor 1 (WCGreen76F, motif top right) and before tutoring from Tutor 2 (WCBlue68, motif bottom right). Bottom left motif is from the same bird at 91 dph after tutoring from Tutor 2 (WCBlue68, motif bottom right).

3.2 Zenk expression

We conducted a nested repeated measures ANOVA with factors Exposure Group (T1 or T2), Hemisphere (Left or Right), and Subregion (Medial or Lateral) to determine if neuronal activation in NCM was dependent on the stimulus to which the bird was exposed at 93 dph. This analysis showed a significant interaction between Subregion and Exposure Group ($F(1,3) = 33.329$, $p = 0.010$). To further investigate this interaction, we proceeded with analysis of lateralization of Zenk expression in each exposure group separately. However, this analysis showed no significant differences for the factors Exposure Group, Hemisphere and Subregion. Therefore, we found no significant differences between Zenk expression in the left and right, medial and lateral NCM due to exposure to Tutor 1 song or Tutor 2 song (Figure 11). This parallels the findings reported in Olson et al. (*in revision*).

In order to determine if there were any correlations between the lateralization of Zenk-positive cells and similarity at 54 and 91 dph to Tutor 1 and Tutor 2, we split the data set into birds exposed to Tutor 1 song and birds exposed to Tutor 2 song and performed correlations between the lateralization ratio of Zenk and similarity to Tutor 1 song at 54 dph, similarity to Tutor 1 song at 91 dph, similarity with Tutor 2 song at 54 dph and similarity to Tutor 2 song at 91 dph. In contrast to Olson et al., we found no significant correlations between lateralized neuronal activation in NCM and similarity to first and second tutor at 54 and 91 dph.

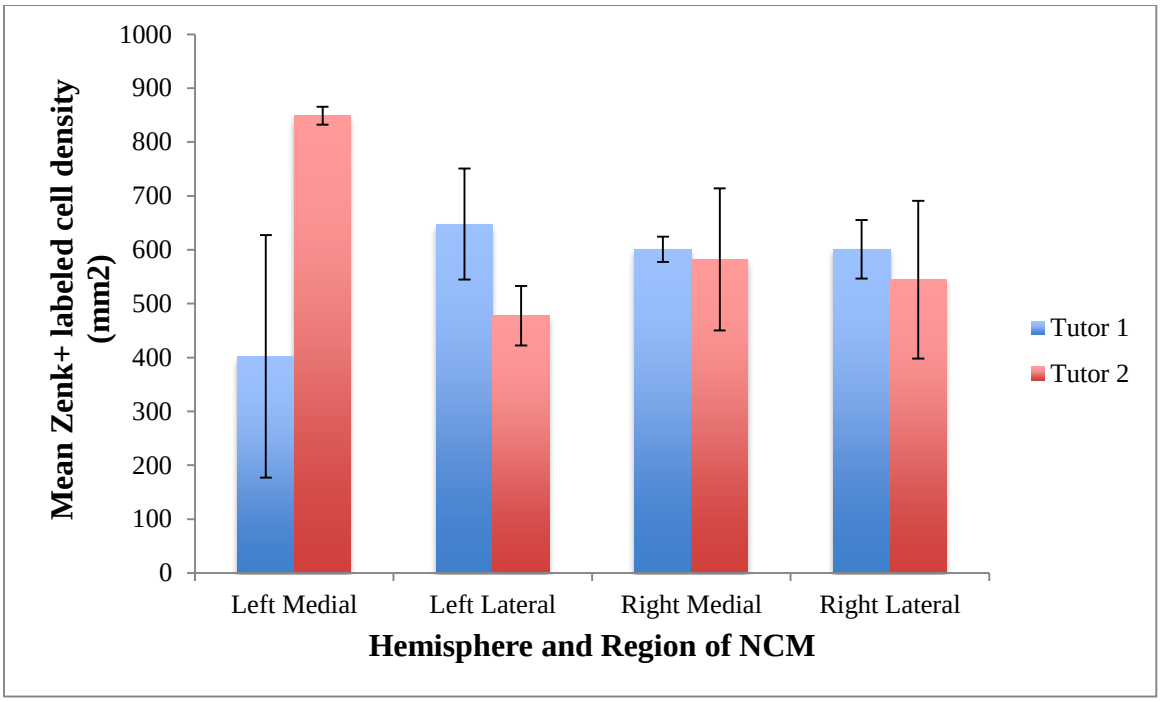

Figure 11: Neuronal activation (number of Zenk-positive neurons) in birds exposed to song stimuli from their Tutor 1 or Tutor 2. Mean (±SEM) number of Zenk-positive nuclei per mm^2 in left and right medial and lateral NCM in response to the song of their Tutor 1 (blue) or the song of their Tutor 2 (red).

3.3 New neurons in HVC

We found no significant difference between mean new neuron densities in the left and right HVC in all birds (Figure 12). In order to determine if the changes in new neuron density in HVC were equilateral, we performed a correlation analysis between the two hemispheres. We did find a significant correlation between new neuron density in left HVC and new neuron density in right HVC (Pearson's r = 0.755, p = 0.019, Figure 13). We then ran correlations to determine if there were any relationships between new neuron addition in HVC and song similarity to first and second tutor. There were no significant differences between new neuron density in left HVC, right HVC, and total HVC to similarity scores at 54 dph and 91 dph to Tutor 1 and Tutor 2 and gain in similarity to Tutor 1 and Tutor 2. Therefore, we were unable to find any significant

relationships between Tutor 1 and Tutor 2 learning and new neuron density in HVC.

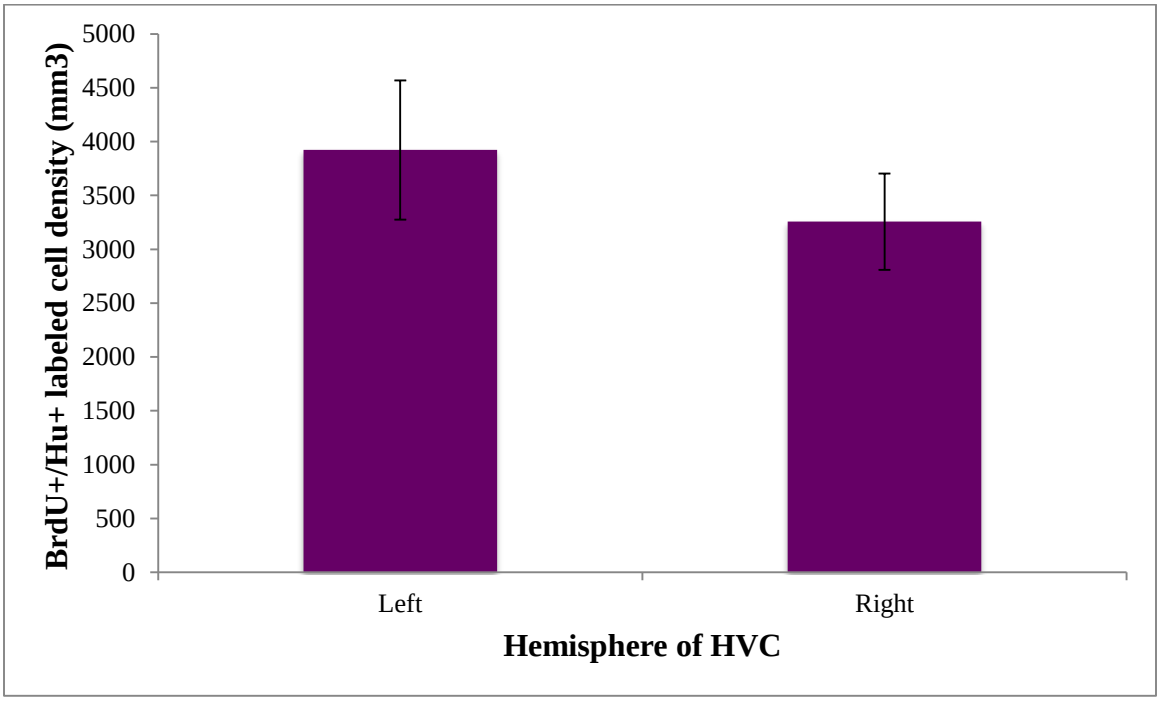

Figure 12: No significant differences between mean new neuron density in the left and right HVC across all birds. Mean BrdU+/Hu+ new neuron density (±SEM) in HVC (per mm^3). New neuron density in left and right HVC was averaged across all birds. No significant difference was found between mean new neuron density in the left and right HVC across all birds.

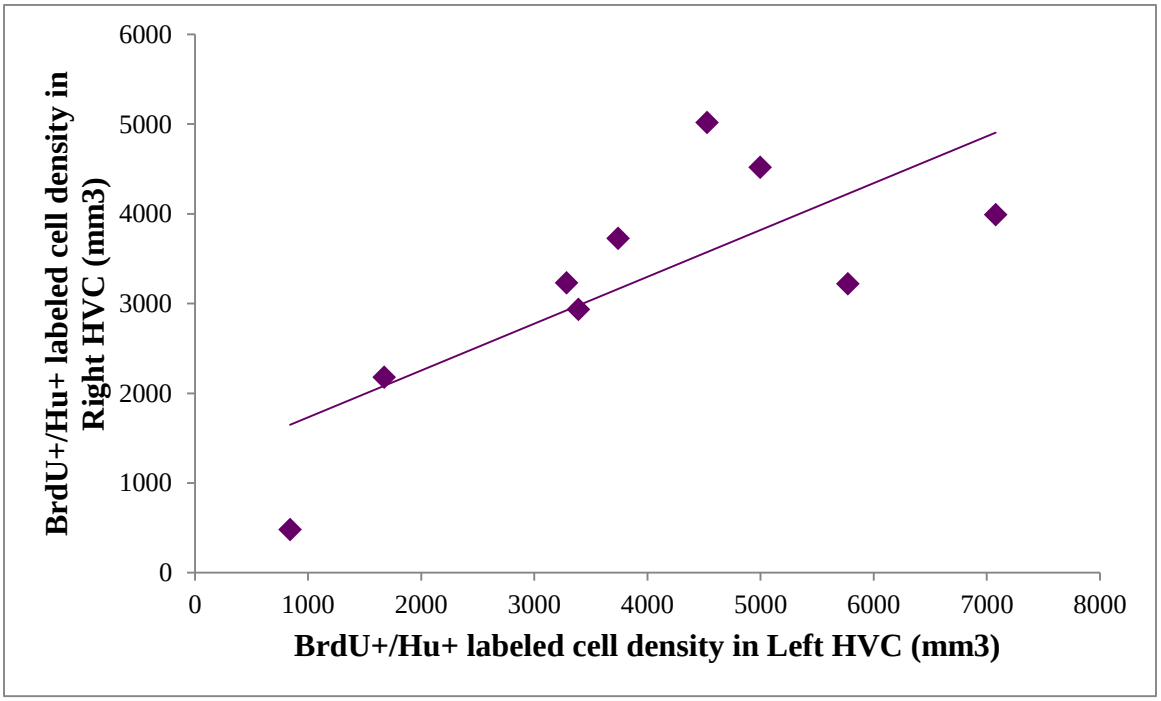

Figure 13: Density of new neurons in left and right HVC increase in parallel. The left BrdU+/Hu+ labeled cell density (mm^3) in HVC is plotted on the x-axis and the right BrdU+/Hu+ labeled cell density in HVC is plotted on the y-axis for each bird. The correlation is significant (Pearson's r = 0.755, p = 0.019).

3.4 New neurons in NCM

We performed a paired samples t-test comparing new neuron density in left and right lateral and medial NCM to determine if there were differences in new neuron recruitment by hemisphere and subregion across all birds. We found that new neuron density in the left medial NCM was significantly greater than new neuron density in the right medial NCM ($t(8) = -2.502$, $p = 0.037$, Figure 14). We found no significant differences in new neuron density between the left lateral and right lateral NCM.

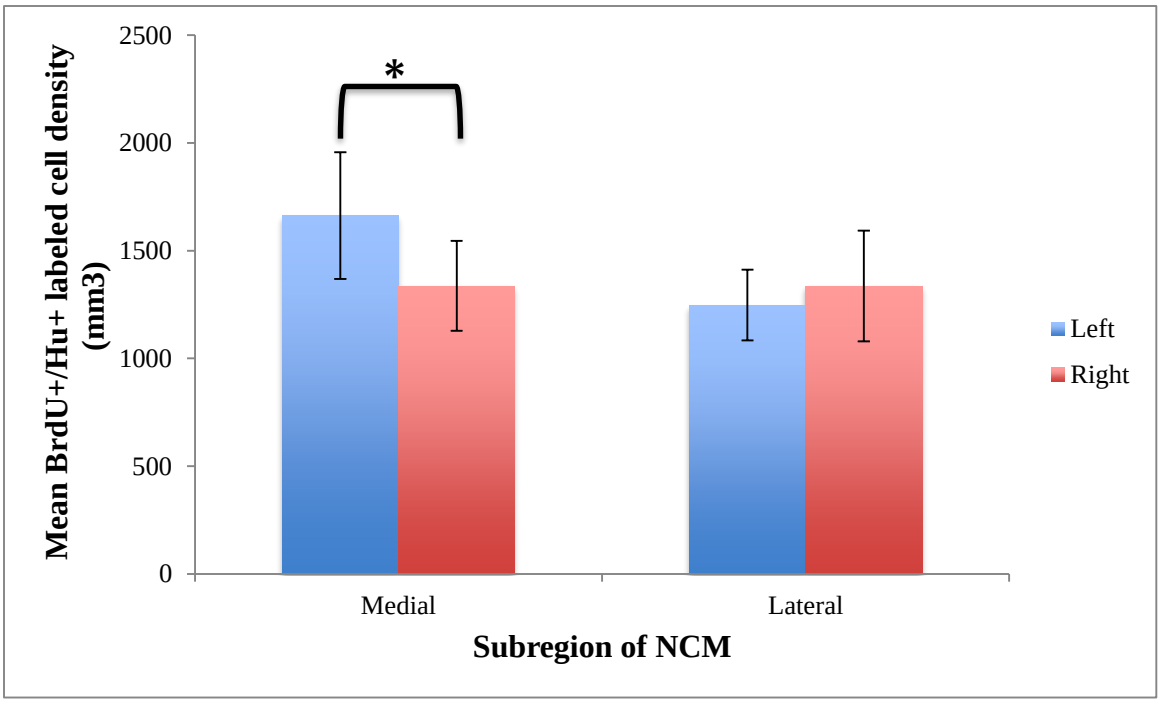

Figure 14: Significantly more new neurons in the left medial NCM compared to the right medial NCM. Mean BrdU+/Hu+ labeled cell density (mm^3) (± SEM) in left and right medial and lateral NCM across all birds. The difference between new neuron density in left medial and right medial NCM is significant ($t(8) = -2.502$, $p = 0.037$).

We then performed correlation analysis between new neuron densities in left and

right medial and lateral NCM against learning scores to determine if there were any relationships between new neuron addition in NCM and gain in similarity to either tutor between 54 and 91 dph (or the duration of time in which birds were exposed to Tutor 2). We found a significant correlation between BrdU+/Hu+ labeled cell density in left lateral NCM and gain in similarity to second tutor ($t(8) = 0.870$, $p = 0.005$, Figure 15). We also found a significant correlation between BrdU+/Hu+ labeled cell density in right lateral NCM and gain in similarity to second tutor ($t(8) = 0.753$, $p = 0.031$, Figure 15). We did not see any significant correlations with new neuron density in left and right medial NCM and gain in similarity to the second tutor.

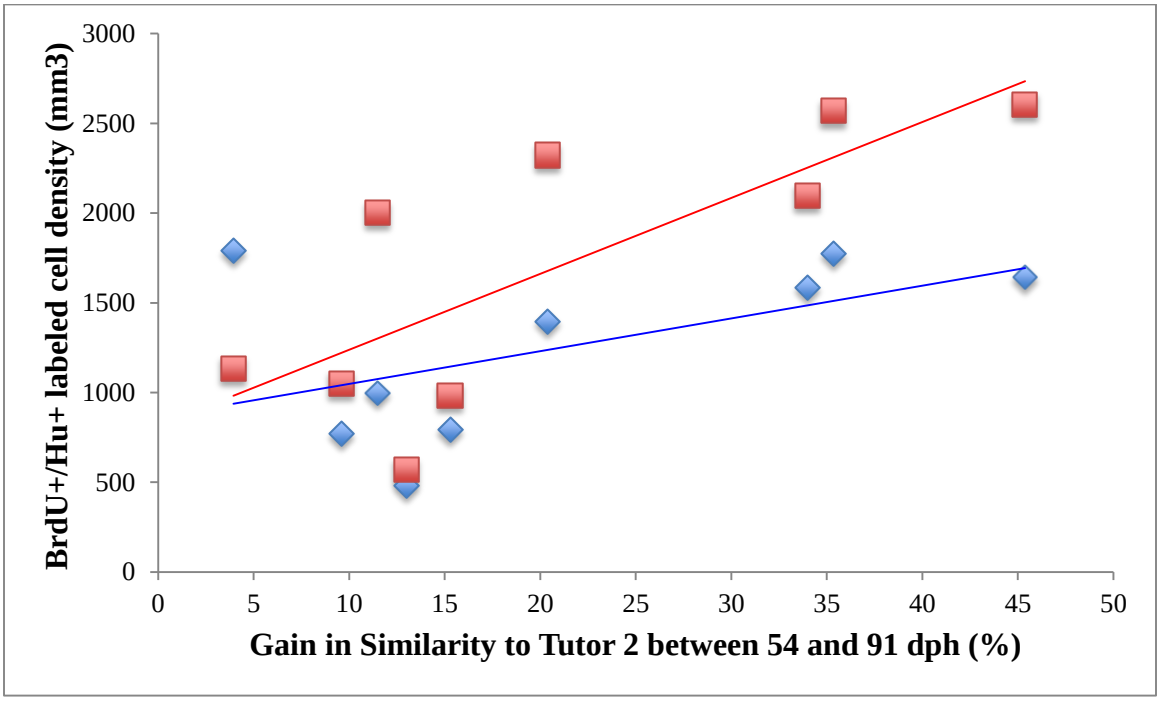

Figure 15: The more new neurons added to the left and right lateral NCM, the more the birds gained in similarity to Tutor 2. BrdU+/Hu+ labeled cell density (mm^3) in left and right lateral NCM on y-axis against gain in similarity to Tutor 2 between 54 and 91 dph (calculated by subtracting the tutee's similarity to Tutor 2 at 54 dph from the tutee's similarity to Tutor 2 at 91 dph). Blue diamonds indicate new neuron densities in left lateral NCM and red squares indicate new neuron density in right lateral NCM. The correlations are significant [(for left lateral NCM (blue), Pearson's $r = 0.870$, $p = 0.005$), for right lateral NCM (red), Pearson's $r = 0.753$, $p = 0.031$)].

We then looked at potential correlations between gain in similarity to the first tutor between 54 and 91 dph and new neuron density in left and right medial and lateral NCM, to determine if there were any relationships between new neuron density in NCM and maintenance of first tutor song. We found a significant correlation between BrdU+/Hu+ labeled cell density in right medial NCM and gain in similarity to the first tutor ($t(8) = 0.738$, $p = 0.037$, Figure 16). We found no significant correlations between new neuron density in left medial, right lateral, and right medial NCM and gain in similarity to the first tutor.

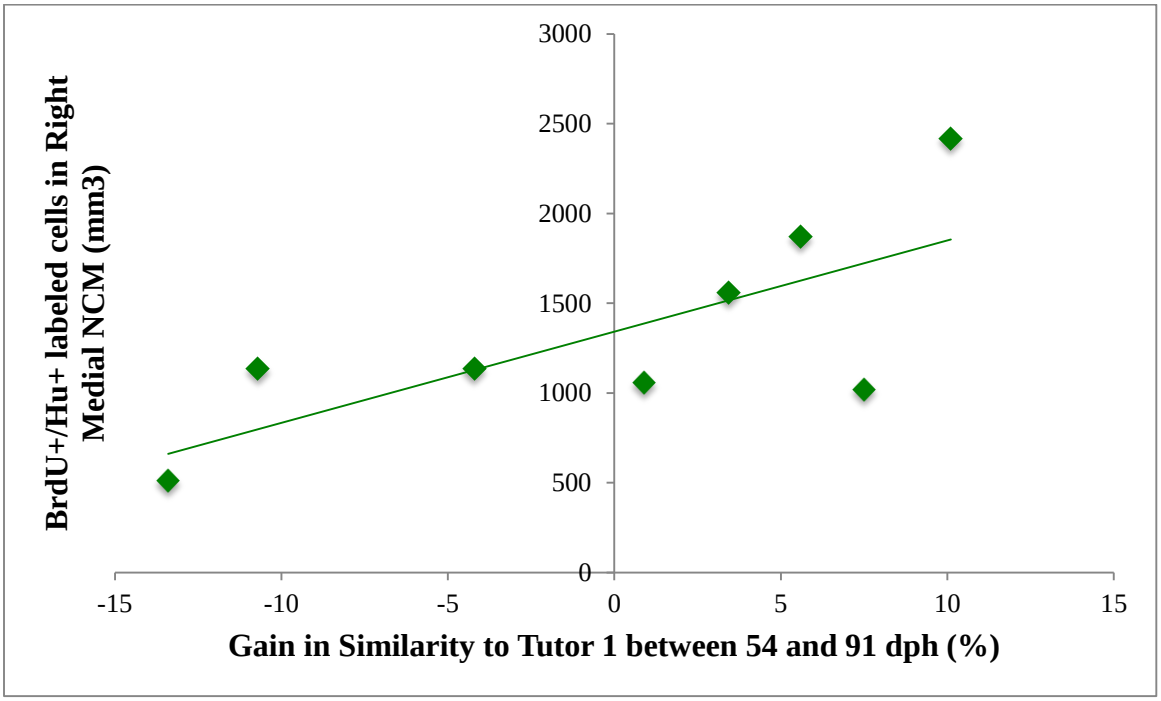

Figure 16: The more new neurons added to right medial NCM, the more the birds gained in similarity to Tutor 1. BrdU+/Hu+ labeled cell density (mm^3) in right medial NCM (indicated by green diamonds) on y-axis against gain in similarity to Tutor 1 between 54 and 91 dph (calculated by subtracting the tutee's similarity to Tutor 1 at 54 dph from the tutee's similarity to Tutor 1 at 91 dph). The correlation is significant (Pearson's $r = 0.738$, $p = 0.037$).

Furthermore, we found a positive trend in the asymmetry of new neuron addition in the lateral NCM and similarity to second tutor song at 91 dph. Asymmetry was

obtained by calculating the lateralization ratio, or the relative difference in BrdU+/Hu+ labeled cell density between the left and right lateral NCM, using the equation (L-R)/(L+R). Although this relationship is not significant, we report it as a positive trend nearing significance (Pearson's r = 0.676, p = 0.066, Figure 17). We found no relationships between the asymmetry of new neuron addition in the lateral NCM and gain in similarity to first tutor song, gain in similarity to second tutor song, and similarity to first tutor song at 91 dph.

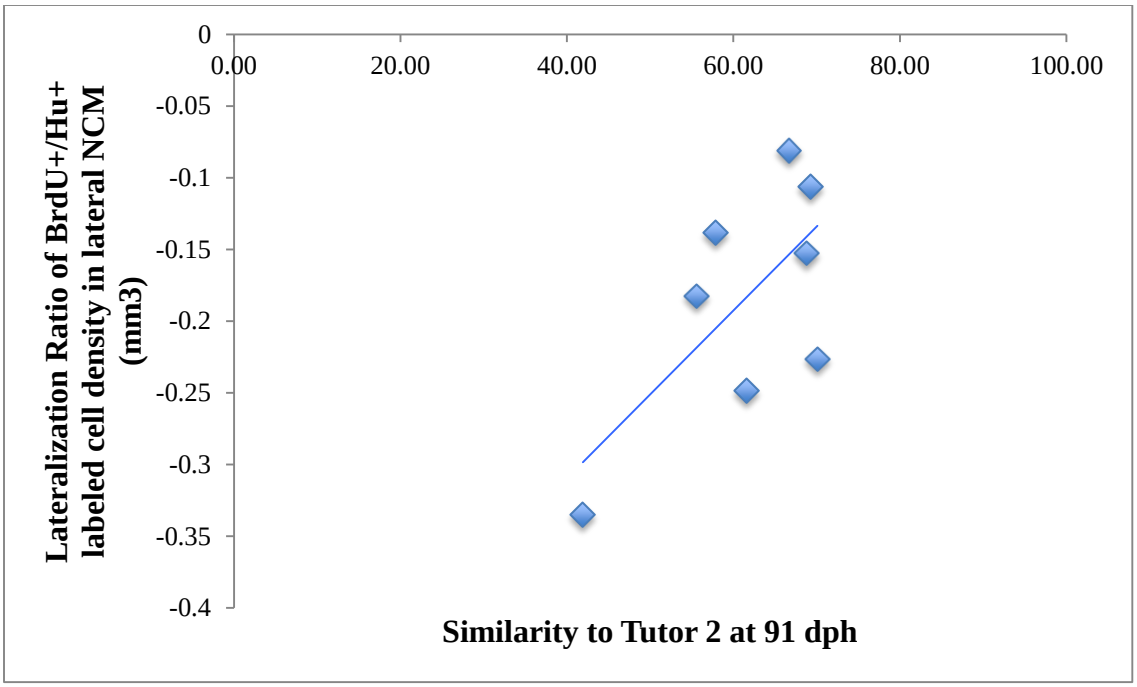

Figure 17: The asymmetry in new neuron addition in lateral NCM is related to the fidelity of imitation of second tutor song at adulthood. Lateralization ratio, calculated by [(L-R)/(L+R)], of BrdU+/Hu+ cell labeled density in lateral NCM plotted on y-axis against similarity to the second tutor song at 91 dph on the x-axis. Correlation is not significant, however we report a positive trend (Pearson's r = 0.676, p = 0.066).

3.5 New neurons recruited for tutor song in NCM

We conducted a nested repeated measures ANOVA with factors Exposure (T1 or T2), Hemisphere (Left or Right), and Subregion (Medial or Lateral) to determine if new neuron recruitment for the first and second tutor song template was dependent on

neuronal activation to first and second tutor song at 93 dph. This analysis showed no significant main effects or interactions. Therefore, we found no significant differences between new neurons recruited for tutor song in the left and right, medial and lateral NCM due to exposure to Tutor 1 or Tutor 2 (Figure 18).

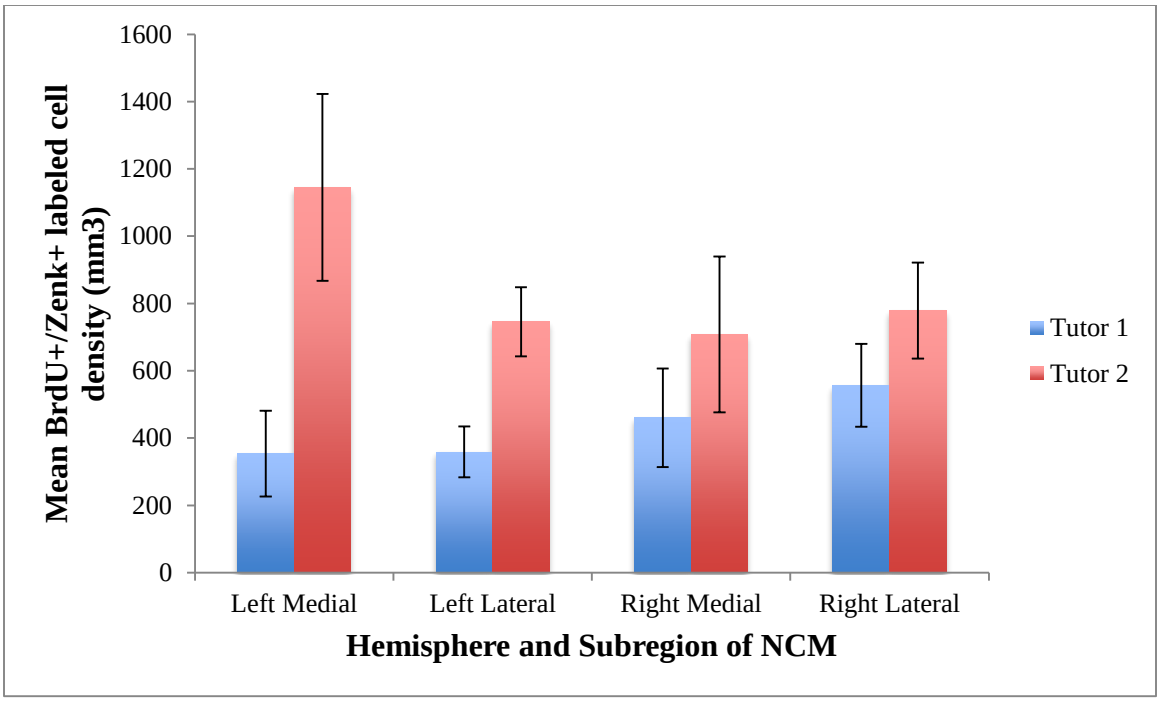

Figure 18: New neurons activated by tutor song in birds exposed to either their first tutor or second tutor in NCM. Mean new neurons activated by first or second tutor song, or BrdU+/Zenk+ labeled cells (± SEM) in left and right medial and lateral NCM. No significant differences in left and right medial and lateral NCM by exposure.

We then performed correlation analysis to determine the relationship between new neurons recruited for tutor song in the left and right medial and lateral NCM and learning scores to first and second tutor. In the group of four birds exposed to second tutor, we found a significant correlation between learning scores to the second tutor at 91 dph and the density of BrdU+/Zenk+ labeled neurons in left lateral NCM, or new neurons that were recruited for second tutor song (Pearson's $r = 0.967$, $p = 0.033$, Figure 19). We

found no significant correlations between second tutor similarity at 91 dph and new neuron density in left medial NCM. Furthermore, we found no significant correlations between similarity to Tutor 2 at 91 dph and new neuron density in the right lateral and medial NCM. We also performed correlations against similarity to Tutor 1 at 91 dph and BrdU+/Zenk+ labeled cells in left and right medial and lateral NCM in birds exposed to their first tutor song; we found no significant correlations in any of these measures.

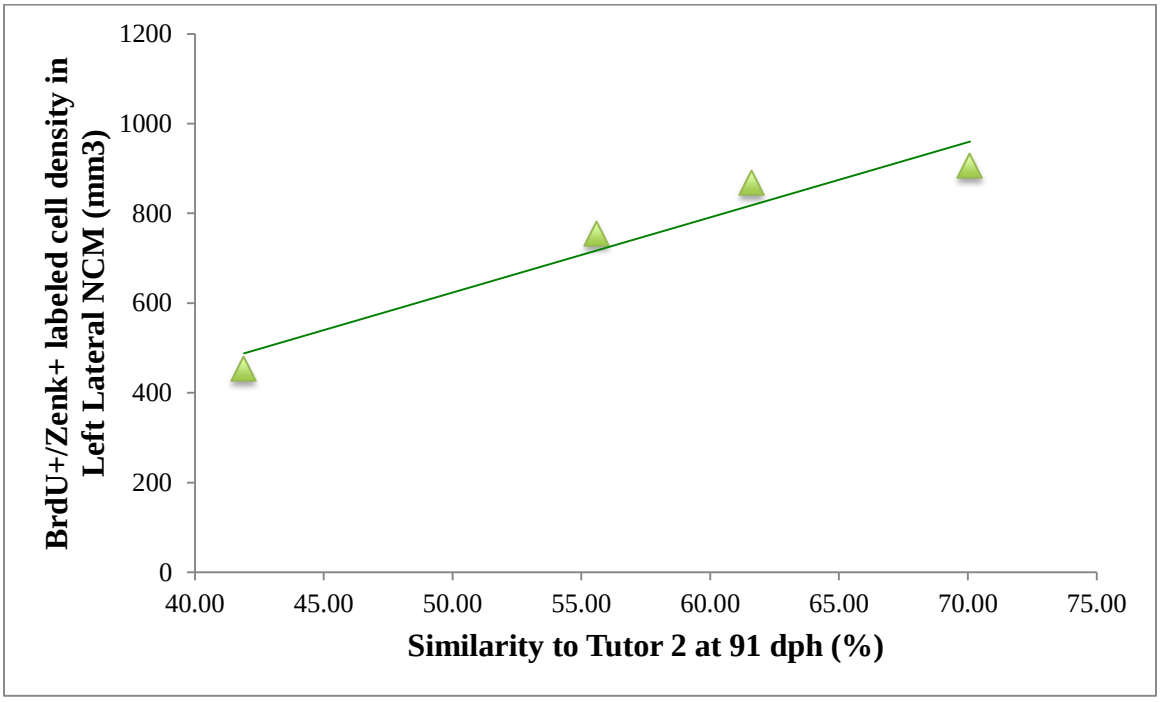

Figure 19: The more similar the bird was to its second tutor at 91 dph, the more new neuron recruitment in the neural representation of second tutor song in birds exposed to Tutor 2. BrdU+/Zenk+ labeled cell density in left lateral NCM (green triangles) on y-axis against similarity to Tutor 2 at 91 dph. The correlation is significant (Pearson's r = 0.967, p = 0.033).

4. DISCUSSION

In this thesis, we exposed male zebra finches to two different tutors at different points in development and examined levels of new neuron recruitment in NCM and HVC at adulthood, in order to determine how new neurons in auditory and motor regions contribute to the acquisition of a second song. Neurogenesis in NCM, an area in the zebra finch brain hypothesized to be the neural substrate for tutor song representation, enabled behavioral plasticity, in that sequentially-tutored zebra finches with more new neuron addition in NCM were better learners of a novel, second tutor song. Furthermore, in the left lateral NCM, we found that greater recruitment of new neurons in the second tutor song template resulted in more successful acquisition of the second tutor song. These findings confirm our hypothesis, and demonstrate that neurogenesis allows for a region to become behaviorally flexible by providing new, plastic neurons that can form a representation of novel auditory information. We did not find any relationships between new neurons recruited in HVC and similarity to either the first or second tutor. This suggests that HVC neurogenesis in sequentially tutored birds does not underlie behavioral plasticity, in maintaining first tutor song amidst a novel auditory environment or gaining second tutor song.

Zebra finches can learn from two different tutors in development

As previously demonstrated by Olson et al. (*in review*) and Yazaki-Sugiyama and Mooney (2004), we found that sequentially-tutored male zebra finches can learn the songs of two different tutors to which they are exposed at different times in development. Learning was evident, as birds significantly learned the song of their first tutor compared

to the song of a novel bird (their second tutor) at 54 dph, and birds significantly learned the song of their second tutor, as shown by the significant difference between similarity to the second tutor song at 54 dph and similarity to the second tutor at 91 dph. This reflects that zebra finches exhibit incredible behavioral flexibility in their ability to adapt to a novel auditory environment, as demonstrated by learning the new song. The sensitive period for zebra finch song learning is estimated as between 25 and 65 dph (Braaten, 2010). Zebra finches in our experiment may have been able to successfully learn a second, novel song, because the second tutor exposure period was between 55 and 65 days, which just marks the end of this sensitive period. In a sequential tutoring pilot experiment conducted at Wellesley College in the summer of 2015, we exposed male zebra finches to a first tutor between days 44 and 49 and to a second tutor between days 68 and 73. We found that male zebra finches significantly learned from the first tutor; however, we found no evidence overall of learning from the second tutor (See Appendix, Figure 18). Despite the experimental differences between the present experiment and the 2015 experiment, including injection times and period of tutor exposure, the difference in overall second song learning between the two experiments provides evidence for a sensitive period for second song acquisition. The sensitive period of 25 to 65 days post hatch for acquiring a single tutor song seems to also hold in sequentially tutored birds, as zebra finches tutored between 55 and 65 days post hatch significantly learned the second tutor song, while zebra finches tutored between 68 and 73 days post hatch, which is beyond the hypothesized sensitive period, showed no evidence of second song acquisition.

Neuronal activation in NCM to first and second tutor song is not related to similarity to first and second tutor song

Although the behavioral data in the present experiment paralleled that found in Olson et al. where birds significantly learned from the first and second tutor, we did not find the same mirrored patterns of lateralization of neuronal activation, measured as the relative difference in the number of Zenk positive neurons between the left and right NCM, in response to first and second tutor song. This may be a result of sample size; the present experiment was conducted on 9 birds whereas Olson et al. included 18 birds for analysis. Therefore, adding more birds to the experiment may minimize the variation in the data set and allow us to observe relationships between neuronal activation and first and second tutor song similarity. Because the correlations found in Olson et al. between neuronal activation to first tutor song and similarity to first tutor at 91 dph and between neuronal activation to second tutor song and similarity to second tutor at 91 dph were significant at $p = 0.001$ and $p = 0.003$ respectively, we expect these correlations to become apparent once more birds are added to the data set (Olson et al., *in review*). Furthermore, our methods may present a reason for the discrepancy. In Olson et al., stained sections were imaged under a light microscope and a single, surface-level image was taken. Cell density was obtained by counting the cells in these single images and then dividing by the area. In the present experiment, we used a confocal microscope to take 15-slice z-stacks of individual brain sections and then collapsed the stacks to obtain a single image, from which we then obtained an area density. In order to more accurately compare the findings of the two studies, our sections should be stained with Zenk and then imaged under the light microscope, using the same method as Olson et al. in order to minimize variation in methodology and allow for more direct comparison.

New neuron addition in HVC is not related to first song maintenance and second song acquisition, nor is it lateralized

Previous studies demonstrate that new neuron addition in zebra finch HVC underlies song maintenance (Adar et al., 2008; Pytte et al., 2011; Pytte et al., 2012; Walton et al., 2012). In our sequentially-tutored birds, we did not observe any relationships between new neuron addition in HVC and song similarity to Tutor 1 and Tutor 2. A 2015 experiment conducted by Vallentin and colleagues found that HVC_{RA} neurons that controlled the output of learned song elements were inhibited by HVC interneurons, while neurons that controlled the output of song elements that were yet to be learned were less inhibited by HVC interneurons (Vallentin et al., 2015). For this reason, HVC activity related to first tutor song may be mainly inhibitory, resulting in reduced new neuron recruitment to encode the motor program for elements of the bird's own song learned from the first tutor. Instead, synapses between HVC_{RA} and RA neurons underlying elements of first tutor song that are maintained are simply strengthened, leaving no need for new neuron incorporation. This suggests that changes in neuronal control of motor production in HVC may be a result of overwriting rather than new neuron recruitment. Instead of recruiting new neurons, neurons in the HVC that control the motor output may simply be overwritten in response to song changing as a result of exposure to the second tutor song because the bird is still in the sensorimotor phase and HVC neurons may still be plastic as a result. An experiment conducted in adult male zebra finches found that it takes at least 3 weeks for new neurons in HVC to be incorporated into circuits for motor control (Tokarev et al., 2015). Therefore, the existing HVC neurons may still be susceptible to novel input and the new HVC neurons that we labeled with our injections may not yet be mature enough to be recruited. If this is the

case biologically, then we should not expect to see any relationships between new neuron addition to HVC and success of maintaining first tutor song or gaining second tutor song.

We also examined lateralization in new neuron addition in HVC to determine if the asymmetric neurogenesis found in NCM was also exhibited in HVC. In comparing new neuron density in the left and right HVC, we found no significant difference between the two hemispheres. However, we found that new neuron density in left and right HVC is significantly correlated, which suggests that an increase in new neuron density in HVC is complimentary in the left and right hemisphere. Bilateral recruitment of HVC in motor control may underlie the lack of lateralized new neuron addition observed in the region. An experiment by Wang and colleagues (2015) found that left and right HVC rapidly alternate control of RA during singing, which indicates that hemispheric coordination is achieved by alternating motor dominance between the two hemispheres. Furthermore, experiments employing unilateral HVC cooling demonstrate that temporal control is synchronized between the two hemispheres (Long and Fee, 2008). Unilaterally cooling the left and right HVC does not result in song degradation, which indicates that both hemispheres contribute equally to control song timing (Long and Fee, 2008). Therefore, although response to song stimuli in auditory regions may be lateralized based on salience, neuronal control of motor production may lack lateralization as both hemispheres contribute in a parallel and complimentary manner, resulting in equal numbers of new neurons added to the left and right HVC.

New neuron addition in left and right lateral NCM is related to the extent of learning from the second tutor

We found that the new neuron density in both the left and right lateral NCM was

correlated with gain in similarity to the second tutor song. Previous studies examining response selectivity to auditory stimuli demonstrate lateral NCM may be the subregion in which the tutor song template is represented (Bolhuis et al., 2000; Terpstra et al., 2004). In this study, after learning the first tutor song, birds were presented with a second tutor song and therefore a new opportunity for learning and encoding of a tutor song template. Consequently, new neuron recruitment in the lateral divisions of NCM may be correlated with second tutor song learning because there is a greater demand for processing and encoding tutor song. Plasticity, as provided by new neurons that have not yet been recruited for other song processing, enables greater behavioral flexibility in a novel auditory environment and successful learning and memory for second tutor song. The contribution of neurogenesis in learning and memory in this context supports findings from other species, specifically rats. Previous experiments examining adult neurogenesis in rats shows that learning augments neurogenesis and conversely, neurogenesis enables learning and memory. Learning stimulates not only the proliferation of new neurons but also their survival, most likely so that new neurons can be incorporated into functional circuits to subserve and maintain what was learned (Gould et al., 1999; Leuner et al., 2004; Epp et al., 2007). Furthermore, blocking neurogenesis in rats through methylazoxymethanol acetate (MAM), an antimitotic agent that is toxic to proliferating cells, or x-ray irradiation, which selectively ablates new neurons, results in reduced ability for learning and memory, which suggests that new neurons are indeed incorporated into functioning memory networks (Shors et al., 2002; Snyder et al., 2005). As they mature, new neurons are more likely than old neurons to be recruited in networks for spatial memory, which suggests that the plasticity of new neurons provides an

advantageous substrate for learning that is not found with mature neurons (Kee et al., 2007). The evidence in rat experiments suggests that new neurons contribute to hippocampal learning and memory by potentially providing a greater capacity for memory storage. We found an analogous contribution of new neurons to learning and memory in NCM, a neural substrate for tutor and conspecific song memory networks. The introduction of second tutor after significant learning of the first tutor may result in increased neurogenesis due to a greater demand for behavioral flexibility. At the point of second tutor introduction, elements of the first tutor song have already been learned, which suggests that an engram for the first tutor song is already established. Thus, the available space to now encode a novel song is limited; therefore, new neurons potentially increase the memory capacity of NCM by providing a flexible substrate for a new network. In both rats and zebra finches, in the present experiment, it appears that new neurons provide a substrate for new memory networks when the memory capacity of a region is already in use for other tasks (Gould et al., 1999; Leuner et al., 2004; Epp et al., 2007; Kee et al., 2007). With the addition of new neurons that allow for greater encoding of tasks and behavior, animals are better able to adapt to a novel environment, such as a new tutor as in our experiment.

New neuron recruitment in memory networks for second tutor song in left lateral NCM is related to greater similarity to second tutor song by adulthood

In NCM, we found a significant correlation between new neuron recruitment in the template for second tutor song in the left NCM and fidelity of second tutor imitation at adulthood. We did not find the same correlation with second tutor similarity in right NCM or between left and right NCM and fidelity of first tutor imitation at adulthood. Our

findings supports our original hypothesis that the left NCM may be involved in storing a representation of the second tutor song as a result of new neuron addition to the region. The more new neurons recruited for the second tutor song memory in the left NCM, the more similar the bird's adult song is to the second tutor song. Therefore, neurogenesis may underlie behavioral flexibility in the left NCM, as represented by similarity to the second tutor at adulthood, but not in the right NCM.

Previous studies conducted in adult male zebra finches demonstrate that novel auditory stimulation induces left-lateralized processing in NCM. Zebra finches raised in a conspecific environment exhibit right-lateralized processing of conspecific song in NCM at adulthood; however, when exposed to just 4 days of novel, heterospecific song, song processing in NCM switches hemispheres so that left-lateralized processing of song is exhibited (Yang and Vicario, 2015). In a separate experiment, absolute responses and adaptation rates to a novel auditory stimulus were greater and faster, respectively, in the left NCM, in zebra finches that had been successfully trained to distinguish between auditory stimuli (Bell et al., 2015). Therefore, birds that are faster to adapt to a novel auditory environment rely on left-lateralized processing in NCM. In the present experiment, the second tutor song template in the left NCM of birds that more successfully adapt to the novel auditory environment (measured by the fidelity of imitation to the second tutor at adulthood) incorporates more new neurons. Because new neurons are plastic and excitable compared to mature neurons, the recruitment of new neurons in the second tutor template in left NCM may correlate with the rate of adaptation, or learning (Aimone et al., 2011). A bird that adapts faster and more efficiently to the novel auditory environment and thus learns more elements from the

second tutor song may incorporate more new neurons in the second tutor template in left NCM as a result. Exposure to a novel auditory environment may result in different responses in the left and right NCM, with a more plastic response exhibited by the left NCM that allows for left-dominant recruitment of new neurons in the second tutor song template.

In both humans and zebra finches, left hemisphere regions involved in vocalization processing are plastic long after the sensitive period has ended (Newmand-Norlund et al., 2006; Hull and Vaid, 2007; Tsoi et al. 2014). In a meta-analysis examining neural activation in response to language in bilingual humans, lateralization of the second language was dependent on the age at which the language was acquired (Hull and Vaid, 2007). Humans who acquire a second language before 6 years of age exhibit bilateral activation to both languages, whereas those who acquire a second language after 6 years of age exhibit left-lateralized activation to both languages (Hull and Vaid, 2007). Furthermore, single-language speakers trained to learn a made-up second language called "Wernickese" exhibit left-lateralized activation in Broca's area in response to hearing Wernickese that is dependent on proficiency with the language. The more subjects adapted to the novel auditory environment and learned the second language, the greater left dominant activation to Wernickese. This left-lateralized, proficiency-dependent response found in humans exposed to multiple languages parallels the findings in the present experiment and suggest that hemispheric plasticity is dependent on both hemisphere and experience. Lateralization-dependent changes in plasticity as a result of novel auditory stimulation may result in lateralization-dependent recruitment of new

neurons in memory networks for novel song, which contribute to more successful acquisition of novel song and greater adaptation to the auditory environment.

Limitations and Future Directions

There are several limitations to our study, which must be taken into account when interpreting the conclusions of the present experiment. First, there is currently no published study examining the timeline of new neuron birth and migration to NCM and HVC in juveniles. Therefore, it is difficult to create an experimental paradigm that allows for proper injection times and sufficient exposure to the first and second tutor for successful learning. We injected 2 weeks before exposure as a result of an email conversation with Carlos Lois, a scientist at Caltech who studies neuronal migration in adult zebra finches. However, the trajectory of neurogenesis in the juvenile zebra finch is likely different, so we may not be catching the peak time of new neuron recruitment in these regions. A pilot experiment should be conducted with varying injection times throughout development so that we can better understand the trajectory of neuronal development in juvenile zebra finches. Furthermore, we can only study new neuron recruitment for the second tutor song in the present experiment and cannot address the potential contribution of second tutor song overwriting first tutor song in neurons recruited for the first song. In Spring 2015, we conducted an experiment in which we used two different new neuron markers, EdU (5-ethynyl-2'-deoxyuridine) and BrdU. We injected one marker before Tutor 1 exposure and the other marker before Tutor 2 exposure so that we could label two different new neuron populations, one recruited for Tutor 1 song learning and one recruited for Tutor 2 song learning (see Appendix, Figure

21). This experimental paradigm was used on 11 male zebra finches. Unfortunately, this timeline only allowed for 5 days of tutoring from Tutor 1 and Tutor 2 and as mentioned earlier, the second tutor period was pushed back to later in development. In this experiment, birds significantly learned from Tutor 1, but did not learn from Tutor 2 (see Appendix, Figures 22 & 23). Therefore, we were unable to answer our original research question of neurogenesis and behavioral flexibility, as the birds did not learn the second tutor song. Furthermore, we were unable to develop a protocol that successfully stained EdU-labeled cells. Brain sections from this experiment were recently processed for BrdU, Zenk, and Hu, and analysis will focus on HVC, rather than NCM. This experiment should be further pursued in the future, with a more successful experimental paradigm, so that we can determine whether overwriting plays a role. Injection stress may also affect not only new neuron counts but also song learning. Honarmand and colleagues (2015) found that new neuron recruitment in HVC was lower in zebra finches that were exposed to early developmental stress, which was induced through moderate food deprivation. Despite the fact that all birds in the present experiment were exposed to injection stress, some birds may have been more vulnerable than others. In order to reduce this effect, the sample size must be increased so that the variation in vulnerability to injection stress can be reduced. Additionally, manual identification of BrdU+/Zenk+ labeled cells and BrdU+/Hu+ labeled cells through the z-stacks also presents an issue. Although the experimenter was blind to the stacks so that counting and consistency was maintained, an automatized method would allow for greater accuracy. Efforts to use several different programs to analyze the stacks were made but none of them proved to work adequately. If these experiments are continued further, designing a computer program to

automatically count cells with the same parameters each time would be essential. Our control data also demonstrates a potential issue with the immunocytochemical protocol or the imaging parameters. We found Hu labeling in sections for which the Hu primary antibody had been omitted during staining. Therefore, further examination of the methods and data set must be conducted in order to determine the source of the error and devise a method for correction. Finally, the sample size of nine birds is too small to make secure conclusions based on the data, and the experiment must be replicated in a larger cohort to make stronger conclusions. Ideally, a future experiment would feature two injection times to account for new neurons recruited for the first tutor and second tutor song, more accurate counting methods, and a larger sample size, so that the results from the present experiment can be validated.

Implications

In this thesis, we demonstrate a correlation between neurogenesis and song learning. In NCM, an auditory region hypothesized to hold the tutor song template, greater new neuron density in the left and right hemisphere was correlated with more successful acquisition of a novel, second tutor song. Furthermore, when more new neurons were recruited in the second tutor song template, the zebra finch's adult song was more similar to its second tutor song. Introducing zebra finches to a novel auditory environment later in development may induce lateralized plasticity in NCM, which affects the extent of behavioral flexibility, as represented by the fidelity of imitation from the second tutor at adulthood. The results of the present experiment add evidence to the growing body of literature that suggests an important contribution of neurogenesis late in

development and in adulthood to learning and memory. Neurogenesis may enable behavioral plasticity by providing novel substrate for developing memory networks. This may allow for efficient development of new memory networks and maintenance of existing memory networks, resulting in successful adaptation to novel environments.

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6. APPENDIX

Control sections were stained with primary or secondary antibody omitted to examine cross-staining. Two sections were stained with everything except BrdU primary antibody (Figure 20A), two sections were stained with everything but Zenk primary antibody (Figure 20B), two sections were stained with everything but Hu primary antibody (Figure 20C), two sections were stained with everything but BrdU secondary (Figure 20D), two sections were stained with everything but Zenk secondary (Figure 20E), and two sections were stained with everything but Hu secondary (Figure 20F).

The image in Figure 20C indicates that although Hu primary was omitted, Hu labeling is still observed. This may be due to a microscopy error or a cross-staining error in the immunocytochemical protocol. The staining and imaging methods must be further examined and tested so that the nature of the error is determined and a method for correcting the error is developed.

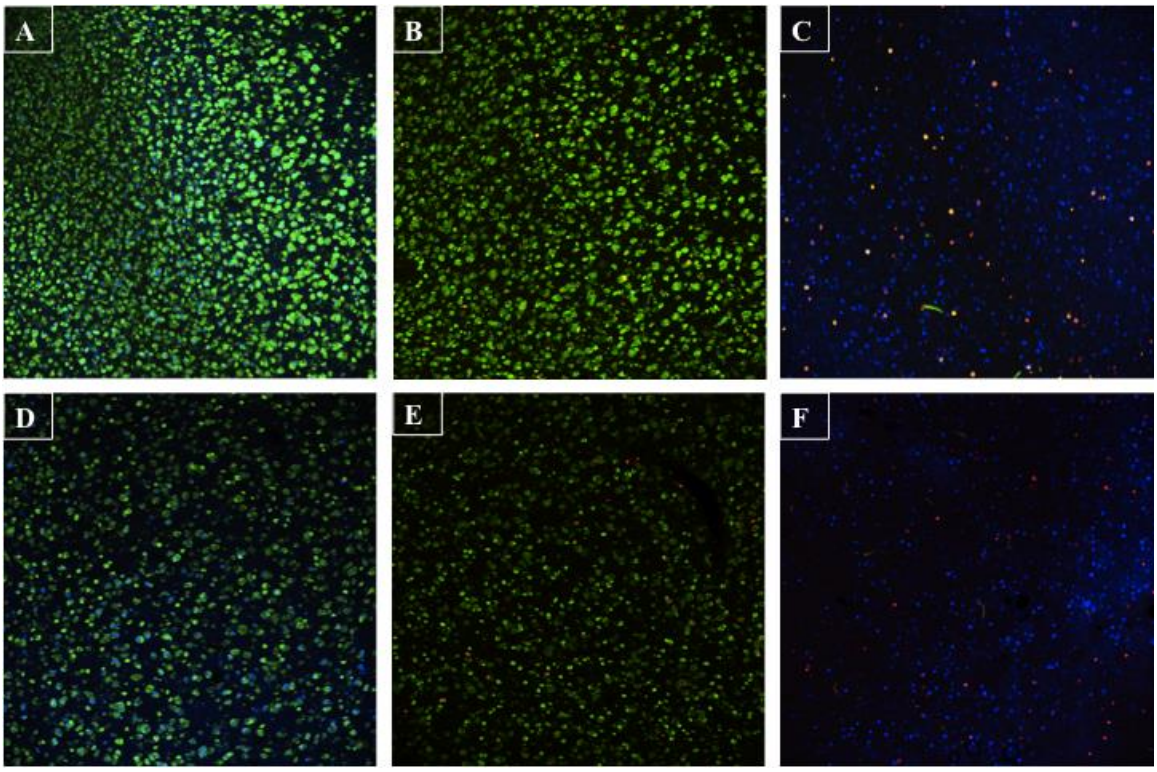

Figure 20: Images of control sections. In A, immunocytochemistry protocol was followed, omitting BrdU primary antibody. In B, immunocytochemistry protocol was followed, omitting Zenk primary antibody. In C, immunocytochemistry protocol was followed, omitting Hu primary antibody. In D, immunocytochemistry protocol was followed, omitting BrdU secondary antibody. In E, immunocytochemistry protocol was followed, omitting Zenk secondary antibody. In F, immunocytochemistry protocol was followed, omitting Hu secondary antibody. For each image, 4-1.01 µm thick slices were collapsed into one image.

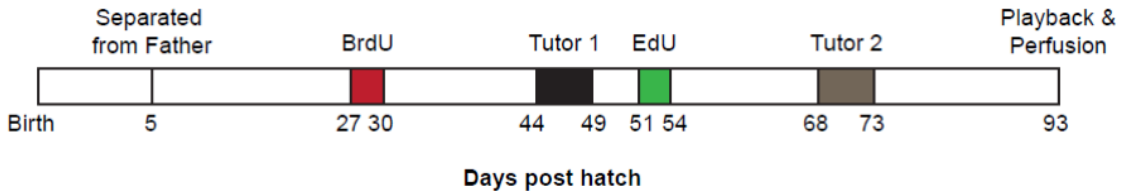

Figure 21: Experimental timeline from Summer 2015 experiment, unpublished pilot. In this experiment, we attempted to label two different new neuron populations, one population recruited during first tutor song learning and one population recruited during second tutor song learning. We injected animals with BrdU between 27 and 30 dph, 3 times per day for 4 consecutive days. We then introduced a live-in tutor, Tutor 1, who lived with the tutee for 5 days. Between days 51 and 54, we injected animals with EdU, in order to label a different population of new cells. We then introduced a second live-in tutor, Tutor 2, who lived with the tutee for 5 days. Finally, at day 93, we exposed the bird to a song stimulus of either their first tutor or second tutor to induce the expression of Zenk; following presentation of stimulus, birds were sacrificed.

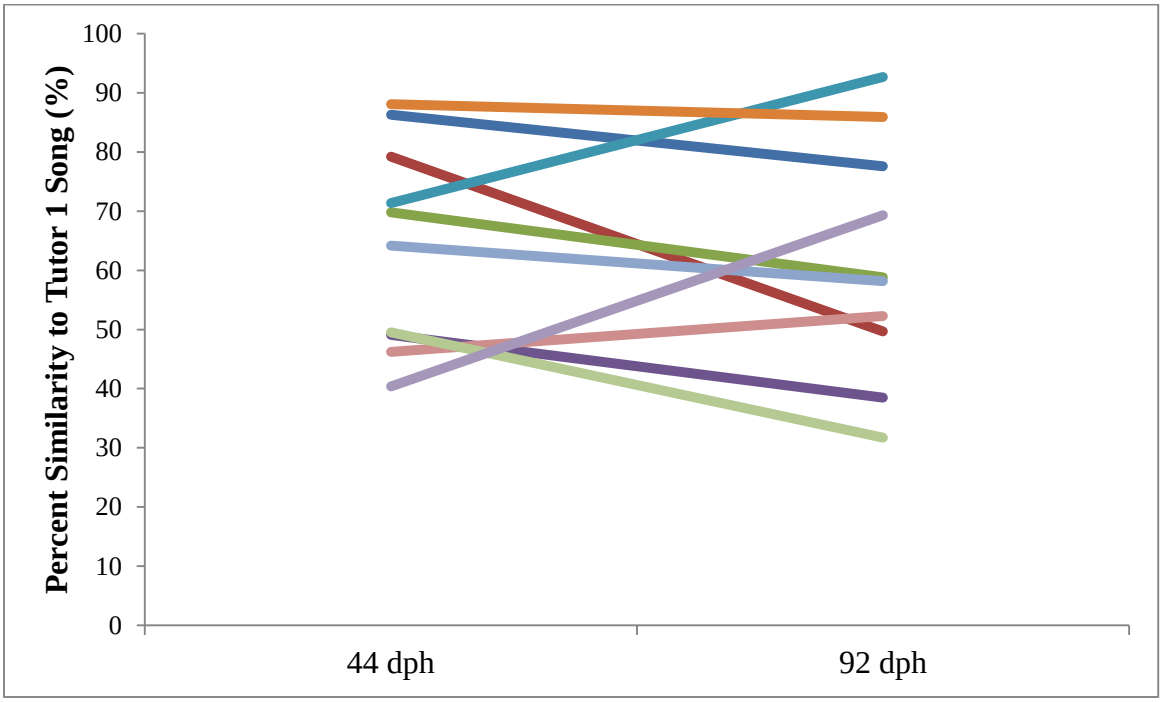

Figure 22: Trajectory of similarity to Tutor 1 in Summer 2015 data set. Each colored line represents a different zebra finch in the data set. Zebra finches significantly learned from Tutor 1 by 44 dph ($p < 0.0001$)

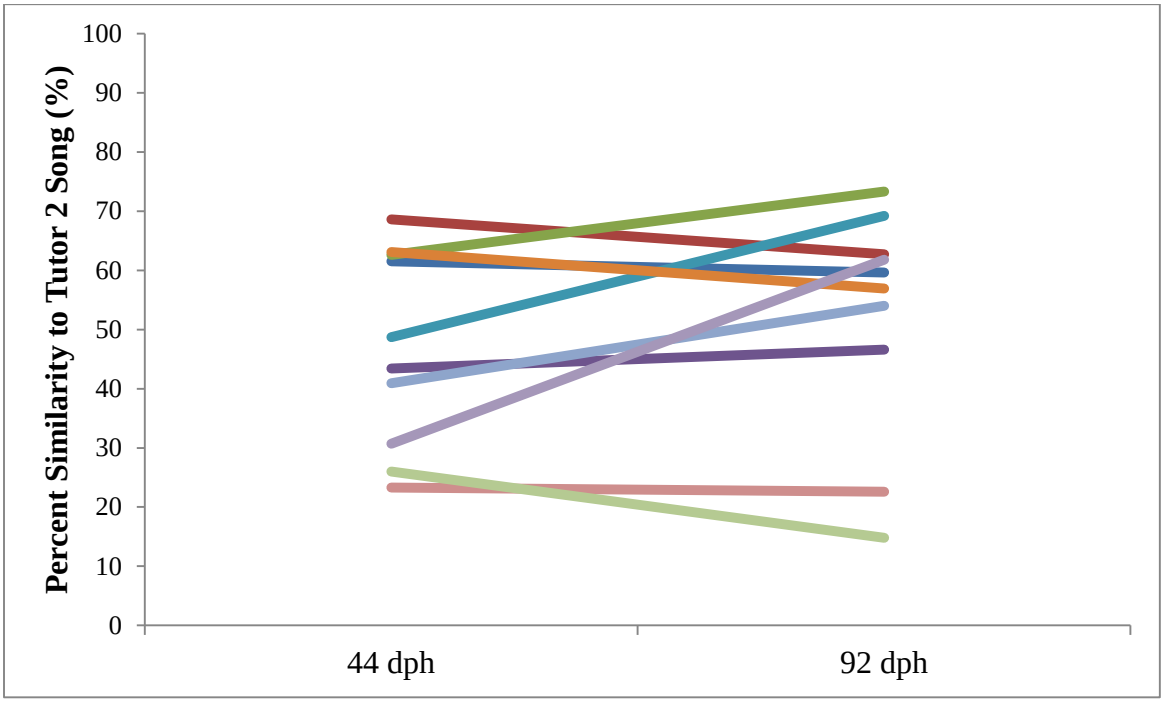

Figure 23: Trajectory of similarity to Tutor 2 in Summer 2015 data set. Each colored line represents a different zebra finch in the data set. Zebra finches did not significantly learn from their second tutor.

PROTOCOLS:

A. INJECTIONS

BrdU Protocol
By Rie Maeda, Fall 2015

BrdU Protocol: Making, Storing and Animal Handling with BrdU

SAFETY: BrdU is toxic. It has teratogenic and mutagenic properties and other severe side effects. Always wear gloves when handling BrdU and make sure to handle it carefully.
*In case of spillage: if the spill is small, clean thoroughly with lots of soap and water and paper towels. Dispose of the paper towels in a plastic bag, label it with "contaminated with BrdU," and then place it in the freezer in the animal facility for incineration. If it is a large spill: leave the room and contact the lab tech and Sharon immediately!

Making BrdU:

**Wear a pair of gloves during every single step of this protocol!

The powder to make BrdU is in the freezer in a clear vial with a green label that says "BrdU, 5 g."

Bring this powder back into lab and weigh out in a small weigh boat using the fine scale.

Also get the TBS-T from the fridge; it is labeled and is in an Erlenmeyer flask on the door.

The ratio of TBS-T to BrdU is 10 mg/ml. So if you are making 1 mL, use 10 mg or 0.01 g on the scale. Use this ratio for all the concentrations that you make.

Add the weighed BrdU powder to a clean vial in the hood. Then add TBS-T to the vial; be careful not to splash this in so that your powder and buffer do not get anywhere.

Cap this vial tightly, label it with "BrdU" and the date you are making it, and vortex for a VERY long time until all BrdU solid has dissolved. This takes a while. Also make sure that the cap is tight; I recommend holding your finger on it closed because there has been a time where the cap came off during vortexing.

Storing BrdU:

Once all is dissolved, store in the fridge at 4 C. This has a shelf life of about 2 weeks so be wary of how long you are using this. If you have extra BrdU put in hood and notify Julia.

Animal Handling with BrdU:

Birds in the neurogenesis project are injected 3x per day for 4 consecutive days. The 3x per day injections must be 8 hours apart. We often do a 7am, 3 pm, 11 pm schedule. Each injection time is 80 ul of BrdU. This is up to 0.08 (or 8 tiny ticks up) on the syringe. *Always be on time for an injection! IF you are late to an injection, take note of how late you were from your scheduled time and make sure to push back 8 hrs from that time and notify the other people you are working with. For example, if you are 1 hour late to the 3 pm injection, all other injections in your series with that bird MUST be pushed back 1 hr. So if you got there and injected at 4pm, the next injection is at 12 and then the next injection is at 8 am and so on. It is crucial that these time frames are kept!

Preparing for an injection: Get a syringe and then a 28 g needle (in the grey box with the grey base). Put the top part of the syringe into the opening of the needle and then push until it clicks and the two parts are connected. Then remove the cap by pulling out the syringe. If the syringe has connected to the needle, then it should not move out when you do this. Only the cap will. *Once you take off the cap, do not put the cap back on!

*Gloves must be worn for every step after this!

Grab the vial of BrdU from the fridge and bring to the hood.
In the hood, suck up 80 ul of BrdU from the needle. **REMOVE ALL AIR BUBBLES!! I recommend sucking up more than you need (more than 80 ul) and then getting all the air out and then pushing it down to 80 ul.
It is CRUCIAL that all the air bubbles are out. If you inject air into any animal, it will die.

Put the BrdU back in the fridge.

Bring the needle to the animal facility.

Turn off the recordings of the bird you are injecting.

Stick a sign on the cooler that says “BrdU injected bird housed here. Do not change cage” Leave this sign up until you clean the cage (this is discussed below)

Take the bird out of the cooler. Get the bird in your palm so that it’s head is near the base of your palm and its tail is on the end of your ring and middle finger. Use your thumb to push back the legs and make sure that the base of the wings are secure. Otherwise, the bird will struggle if its wings are not held in place.

Push the feathers back away from the chest. You can use water or alcohol to move the feathers away – I recommend using water as the alcohol can irritate them, which can make them struggle.

Once the bird is secure and feathers are out of the way, inject in either the left or right pectoral muscle (**make sure to switch sides of injection at each time!)
-Do not inject to high up (towards the head) because the heart is there, you can often see it beating.
-Do not inject to far down as the muscle gets thinner and there is the risk of injecting too deep and hitting something.
*Do not inject too much in the midline where the bone is.

*When you are injecting, make sure the pointy tip of the needle is facing into the chest (not the flatter part!!!) The very sharp edge should go in first, otherwise this will be more painful than it should be.

-Make sure to inject in one smooth motion. Do not go to deep – you should feel some resistance and the needle should go in about halfway.

Once the needle is in, push down the syringe to administer the BrdU very slowly. *Make sure that you are not pushing in the needle as you are pushing down on the syringe.

Once you have administered all the BrdU, place a finger on the injection site and then pull on the needle slowly. **ALWAYS put your finger on the site BEFORE the needle goes out otherwise, the bird will definitely bleed and bruise!!!!!

Apply gentle pressure for about 1 to 2 minutes.

Then gently place the bird back in cage.

Close the cooler and turn the recordings back on.

Dispose of your gloves and dispose of the syringe in the sharps bucket in the rat surgery room (right across from the bird recording room)

Other Notes:

If you are injecting more then one bird, use the same needle and just change the amount of BrdU in your needle. If it's 2 birds, add 160 ul in the needle; 3 birds and its 240 ul and etc.

Cage Handling:

Keep the "BrdU housed bird" sign on the cage for the 4 days of injections AND for 3 days after injections terminate.

3 days after the last day of injections – you can clean the cage.

Remove the sign from the cooler and remove the cage.

Prepare a new cage and put the bird in this cage and then put the bird in its new cage back in the cooler.
With the old cage – this is contaminated with BrdU and must be disposed of accordingly.

Remember – wear gloves when handling this!!

Dump the rest of the food in the food bin into the bottom of the cage.

Remove the perch, water bottle and empty food bin. Put the perch and empty food bin in the bin of soapy warm water next to the sink in the animal facility. Rinse out the water bottle and hang it up on the rack above the sick and put the cap in the bin of soapy water.

Remove the top of the cage and put it in the appropriate place for cleaning.

Get a clear trash bag (found in the animal facility) and dump the paper bottom and rest of the cage contents into this plastic bag (obviously except for the plastic cage bottom). All the food waste and the paper liner should go in this bag.

Place the plastic cage bottom in the appropriate place for cleaning.

Put your gloves in the trash bag and then secure tightly. Get another trash bag and put the secured trash bag in this trash bag. Close the second trash bag.

Label the trash bag with "Contaminated with BrdU" and then dispose of in the freezer for incineration.

LAST NOTES:

1. Always wear gloves when handling BrdU and thoroughly wash your hands after using BrdU!!

2. Be gentle when handling the animals and be as careful as possible when doing the injections. Ensure that this is a painless procedure as possible! Always make sure that the animal is not in pain – that is your number 1 priority. If you think the animal is suffering, notify the lab tech or Sharon.

3. Always make sure to turn recordings BACK ON after you inject an animal and put it back in the cooler. This is easy to forget but if you do, you will lose hours of important recordings.

****IF YOU EVER HAVE ANY QUESTIONS: always ask the current lab tech or Sharon**

B. BEHAVIORAL ANALYSIS

1. Input motifs of tutor and tutee into PRAAT to determine the number of syllables in each motif (Figure #)

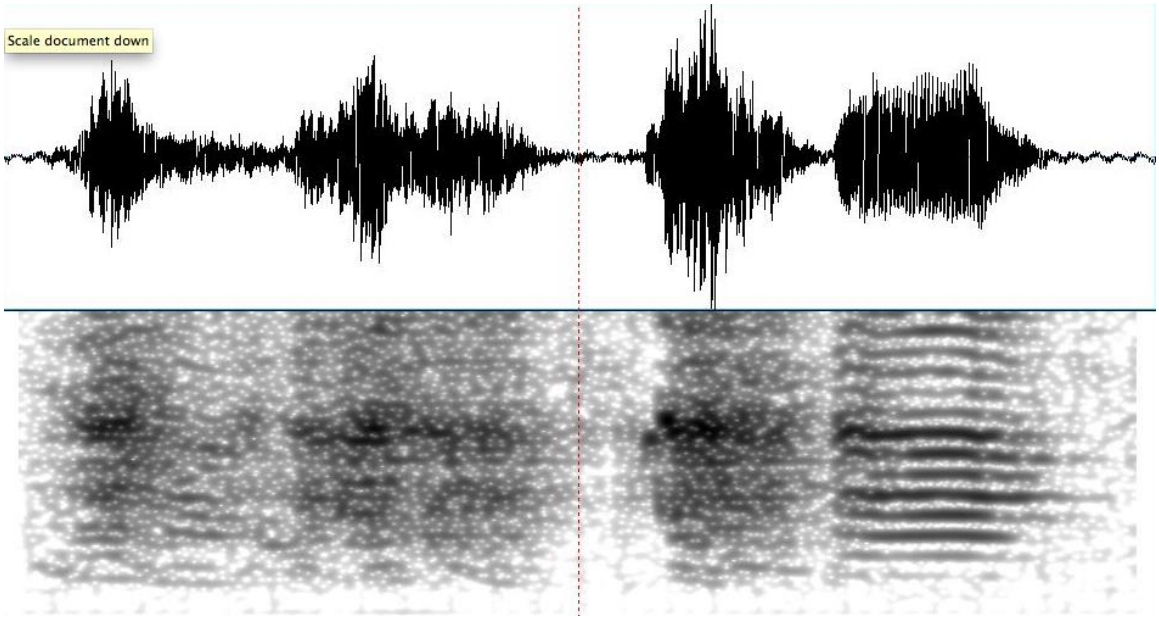

Figure 23: Motif of WCOrange97 has 3 syllables

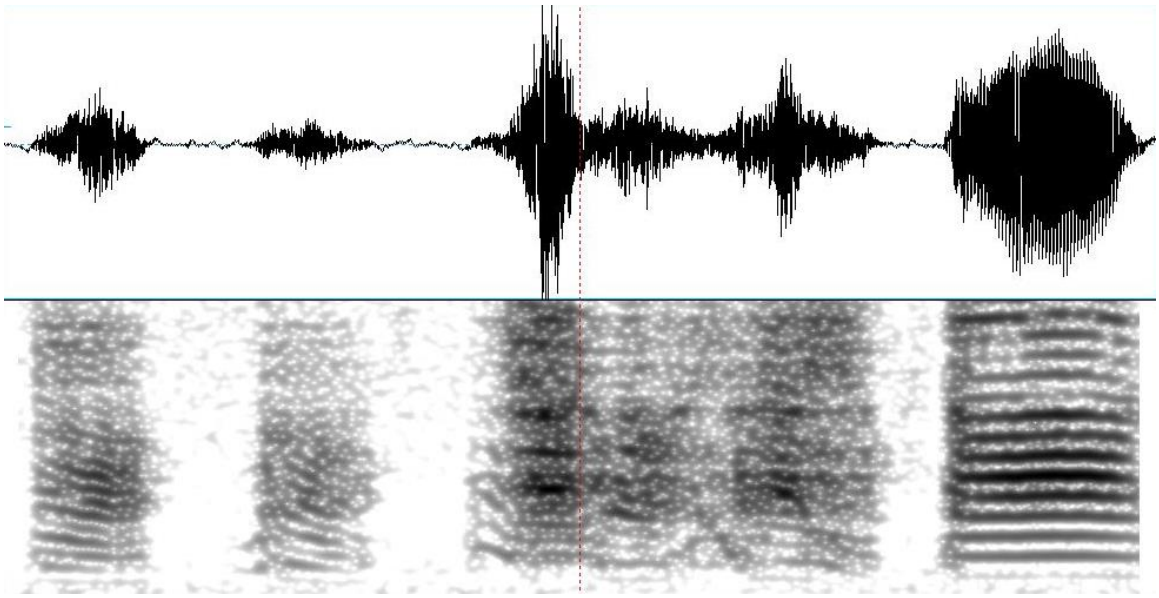

Figure 24: Motif of Blue68 consists of 5 syllables

2. Open SAP and select "Explore and Score."

3. Select the tab labeled "Sound 1" and open the first sound file of the tutor motif. Turn up the amplitude to the maximum intensity and adjust the segmentation so that the

number of syllables in the grey window matches the number you counted in PRAAT (Figure #)

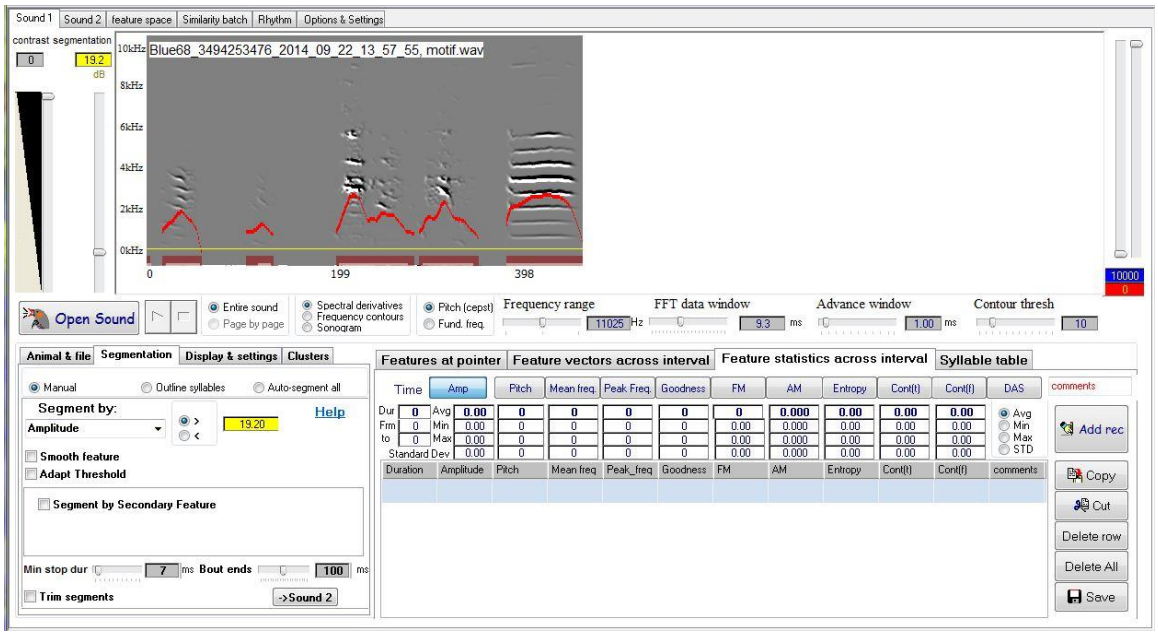

Figure 25: Sound 1 tab with loaded tutor motif, adjusted for the number of syllables counted in PRAAT.

4. Select the tab labeled "Sound 2" and open the first sound file of the tutee motif. Turn up the amplitude to the maximum intensity and adjust the segmentation so that the number of syllables in the grey window matches the number you counted in PRAAT (Figure #)

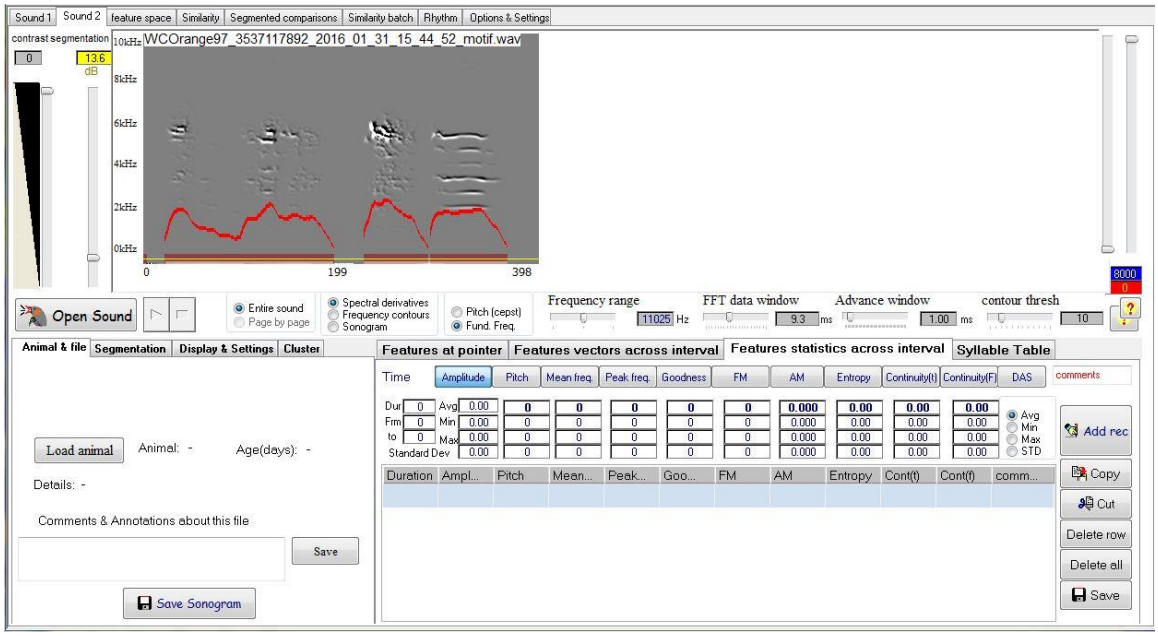

Figure 26: Sound 2 tab with loaded tutee motif, adjusted for the number of syllables counted in PRAAT.

5. Select the tab labeled “Similarity and press the large button labeled “Score.”

6. The program will subsequently compare the tutor and tutee motifs overall (in left bar) and also run a syllable-by-syllable analysis, comparing each motif of the tutor against each motif of the tutee (Figure #).

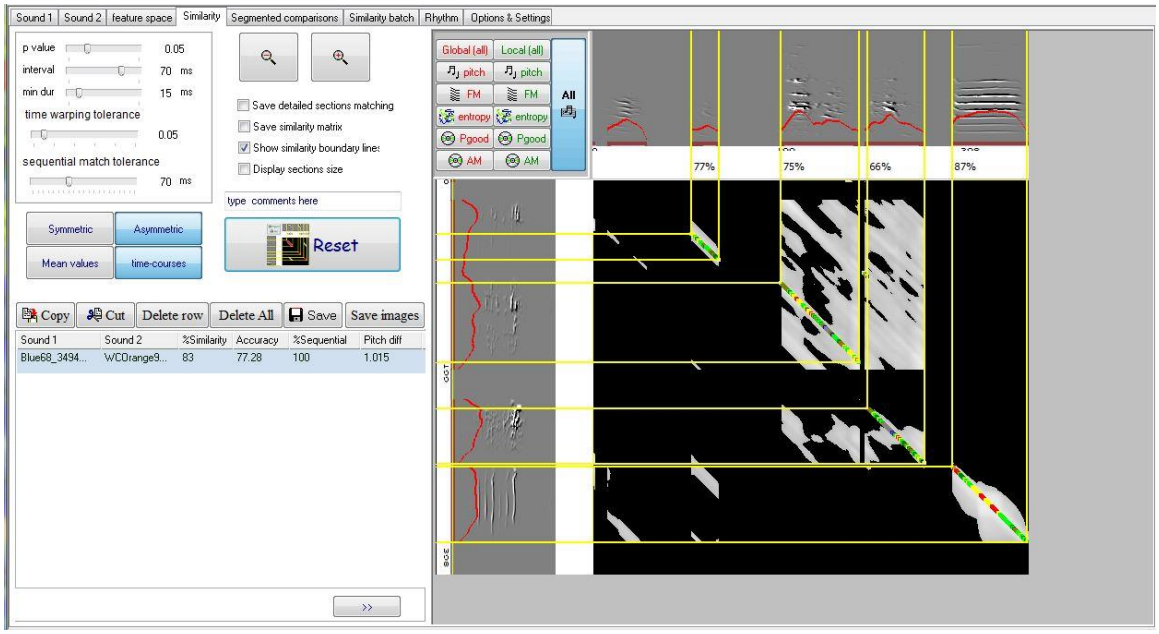

Figure 27: Output of similarity analysis conducted on Sound 1 (tutor motif) and Sound 2 (tutee motif). The % similarity score in the bottom left bar indicates that the two motifs are 83% similar. The syllable-by-syllable analysis indicates 4 syllable comparisons that range from 66 – 87% similarity.

7. Record overall similarity and syllable-by-syllable comparison. Be sure to note which syllable of the tutor was similar to which syllable of the tutee and to what extent (the percent similarity).

8. Repeat this procedure with the remaining 9 tutor motifs and 9 tutee motifs, conducting paired comparisons with different tutor and tutee motifs used each time.

C. IMMUNOCYTOCHEMISTRY

Neurogenesis Project 2015-2016
By Rie Maeda

BrdU-Zenk Double-labeling Protocol

Adapted from Kee Nature Protocols: http://www.franklandlab.com/wp-content/uploads/2007/08/Kee-Nature-Protocols.pdf

****NOTE: When using fluorescent secondary antibodies (so on Day 2 and after) never work with these in the light! Always protect the antibody and sections with the antibody from the light! Use foil and the dark.**

1. The night before your immune, sort your sections from cryoprotectant into PB.

Immuno Day 1:

1. Rinse sections 3x for 5 min. each in 0.1 M PBS on ice on shaker

2. Denature DNA by incubating sections in 1 N HCl for 30 min. at 45 C (use oven in the lab, make sure to turn on oven about an hour before you start so it can get to the right temperature. Do not move the temperature probe!!

3. Take sections out of the oven and neutralize acid by rinsing sections 3x for 5 min. each with 0.1 M PBS on ice on shaker

4. Incubate sections with BrdU (1:500), egr-1 (Zenk) (1:1000), and Hu (1:100) in blocking solution for 48 hrs at 4 C on a shaker.
Blocking solution = 0.1 M PBS, 0.3% Triton X-100 and 2% NGS
For Triton X-100 use, recommend diluting in water and then diluting to PBS solution. Always make sure to pipette up slowly when using Triton-X and remove the pipette slowly from the container of Triton-X as it is very soap-like.

Immuno Day 2:

1. Rinse sections 3x for 5 min. each in 0.1 M PBS on ice on shaker

FOLLOWING STEPS PERFORMED IN THE DARK!!

2. Incubate sections with secondary antibodies CY2, CY3 and CY5 (all at 1:500) for 2 HRS. in the dark at room temperature on the shaker. The antibodies are diluted in 0.1 M PBS with 0.3% Triton-X solution.
Wrap well plates in aluminum and put the brown cardboard box over them while on the shaker. Also turn the lights off.

3. Rinse sections 3x for 5 min. each in 0.1 M PBS on shaker.

4. Mount sections on slides using a soft brush. DO NOT use the special slides for this experiment. Use the normal slides.

5. Carefully dab the water off the slide with a kim-wipe. Wait about 15 to 20 minutes for sections to dry a little.

6. Apply 3 consecutive dots of Fluoromount-G with the tip of a glass pipette to the slide so that they cover your sections.

7. Carefully lower down a coverslip so that your sections are fully covered with Fluoromount-G and there are no air bubbles under the coverslip.
Do this carefully and then once the coverslip touches a bit of Fluormount-G on the slide, let the mounting medium pull the coverslip down.

8. Apply lots of clear nail polish around the edge of the coverslip to seal out the air.

9. Store upright in the dark and allow to dry overnight.

10. The next day, wrap your slide in foil and put it in the fridge.

NOTE ON SECONDARY ANTIBODY STORAGE

Small microentrifuge tubes of CY3 and CY5 are held in a pipette array in the industrial freezer in the hallway of the 3rd floor labs. The array is labeled with Gobes Lab and is on the very bottom shelf of the freezer (in Beltz storage)

Bring foil to the freezer so that you can immediately wrap the antibodies from the freezer holder for storage later. THESE CANNOT BE EXPOSED TO LIGHT!!

When you use this freezer – unlock the freezer by pressing the lever down and pulling it on. Wearing the gloves provided next to the freezer, pull out the door for the lowest shelf. Then look for the array: it is labeled with a pink piece of tape, there is foil under the plastic cover, and the bottom is yellow. It should be on the left side but close to the middle.

Take this out and get out one vial of CY3 and CY5. Immediately wrap these microcentrifuge tubes in foil.

Put the array with the rest of the tubes back in the freezer and close and lock the freezer carefully. Make sure not to keep this open to long otherwise the temperature will drop and an alarm will go off!

Label these antibodies and store at 2 – 4 C in fridge. **These last up to 6 weeks in fridge storage! SO ALWAYS take note of when you took out secondary antibody and do not use it outside of the 6-week period.**

D. IMAGING

Follow settings detailed above in Methods section.

E. COUNTING CELLS

Zenk-labeled cells:

1. Open single image of collapsed stack in Image J.

2. Under Image > Type, convert the image from RGB color to 16-bit image.

3. Under Image > Adjust > Threshold, adjust the threshold of the image so that the brightest Zenk-labeled cells are included in the analysis. Have the image open in color next to the ImageJ version in black-and-white so that threshold is accurately adjusted.

4. Under Process > Filters > Median, apply a radius of 2.0 pixels.

5. Under Process > Binary, select Watershed to separate particles that are close together.

6. Under Edit, select Invert to convert the image from white on a black background to black on a white background.

7. Under Analyze, select Analyze Particles. The Size (inch^2) should be set to 0-Infinity and the Circularity should be from 0.00-1.00. Check the boxes next to Display Results and Summarize.

8. Record the count in the Summary.

9. Divide by the area (775 x 775 micrometers) to obtain an area density.

For BrdU+/Zenk+ labeled cells and BrdU+/Hu+ labeled cells in NCM:

1. Open images of a stack in sequential order in FIJI.

2. Under Images > Stacks, select Images to Stack.

3. Scroll through the stack in order and identify cells that are double-labeled with Brdu and Zenk or BrdU and Hu (depending on which stack you are looking at). I recommend circling the cells you find in each stack using the paint tool, so that you can check back when you go through each image to make sure you haven't already selected a cell.

4. Count the total number of cells in the stack and record this count.

5. Divide by the volume (775 x 775 x 14.70 micrometers) to obtain a volume density.

For BrdU+/Hu+ labeled cells in HVC:

1. Open images of a stack in sequential order in FIJI.

2. Under Images > Stacks, select Images to Stack.

3. Scroll through the stack in order and identify cells that are double-labeled with BrdU and Hu that are within the boundaries of HVC. I recommend circling the cells you find in each stack using the paint tool, so that you can check back when you go through each image to make sure you haven't already selected a cell. The boundary of HVC is clear in images as the cells are much bigger and the staining is brighter.

4. Count the total number of cells in the stack and record this count.

5. To obtain the area of HVC, open one of the images of the stack that includes the whole HVC in ImageJ.

6. Calibrate the image so that the dimensions of the image are 775 x 775 micrometers.

7. In the tool bar, select the Wand (tracing) tool and circle the boundaries of HVC on the image.

8. Under Analyze, select Measure and record the area given.

9. Divide the cell count in step 4 by the volume, obtained by multiplying the area obtained in step 8 by 15.75 micrometers, to obtain a volume density.